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Production d'une activité amylolytique thermostable par *Thermus thermophilus* HB8 suivie du clonage et de l'expression d'une pullulanase de type 1 de *T. thermophilus* HB8 chez la levure méthylotrophe *Pichia pastoris*

Thèse de doctorat
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Fatima Bougrine et Miloud Akassou
pour votre amour, sacrifice et patience.
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Adil, Sabah, Amal, Faïçal et Zahra
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Naël, Nabil, Olaya, et Maya
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Résumé

Le besoin de développement d'amylases thermostables, principalement de pullulanases, s'est accru ces dernières décennies dû à l'importance de ces enzymes dans plusieurs bio-industries. Ce présent projet de recherche s'inscrit dans le cadre général du développement industriel de la production extracellulaire d'une pullulanase thermostable.

Une revue bibliographique sur les avancées technologiques et les défis à relever pour la production extracellulaire de pullulanases thermostables a été réalisée. Cette revue de littérature expose une problématique liée à la production des pullulanases par divers hôtes d'origine et par le biais de production recombinante en utilisant des cellules hôtes comme *E. coli*, *B. subtilis* et *P. pastoris*. Cette problématique se résume dans la difficulté de produire de façon extracellulaire des pullulanases thermostables à haute activité enzymatique, ce qui engendre l'utilisation de plusieurs méthodes de purification coûteuses et souvent difficilement applicables à l'échelle industrielle. La modification structurale de ces enzymes via différentes techniques de *protein engineering* a permis le développement de pullullanases hautement thermostables et/ou ayant une activité catalytique remarquable. Cependant, ces techniques sont coûteuses et nécessitent le développement d'outils de bioinformatique plus efficaces afin de mieux maîtriser le processus de modification.

Une étude expérimentale fut réalisée afin d'évaluer la capacité de *Thermus thermophilus* HB8 à accumuler de façon extracellulaire des enzymes amylolytiques, principalement de pullulanase. Les tests de production initiaux ont indiqué clairement que seulement de très faibles niveaux d'activité amylolytique pouvaient être détectés, à partir d'extraits cellulaires en utilisant le détergent doux non ionique Triton X-100 (TX100). Une stratégie d'optimisation séquentielle, basée sur des techniques statistiques, a été utilisée pour améliorer la production d'activité amylolytique extracellulaire afin d'obtenir des niveaux d'activité industriellement attrayants. L'accent a été mis sur le niveau optimal de la concentration initiale de la biomasse, la composition du milieu de culture et la température pour maximiser l'accumulation d'enzymes amylolytiques. Suite à de tels efforts, l'accumulation d'enzymes amylolytiques extracellulaires a été augmentée de plus de 70 fois, avec des niveaux d'activité de 76 U/mL. La préparation brute d'enzyme extracellulaire a été partiellement caractérisée. Les valeurs optimales de température et de pH ont été trouvées, respectivement, 80°C et 9,0. 100% de l'activité enzymatique initiale a pu être maintenu après 24 h d'incubation à 80°C, ce qui prouve la thermostabilité très élevée de la préparation enzymatique.

Une autre étude sur la capacité de *Pichia pastoris* de produire de façon extracellulaire la pullulanase type 1 de *Thermus thermophilus* HB8 fut réalisée. Les effets de différents paramètres sur la production extracellulaire de la pullulanase ont été déterminés, à savoir les séquences peptides-signal de sécrétion (SP), la présence de Triton X-100 (TX100) à différentes concentrations et à différents temps d'addition, la présence de différents osmolytes naturels, le pH, la température et la source de carbone. La production de pullulanase extracellulaire a augmenté de plus de 40 fois en ajoutant de l'acide K-glutamique (0,40% (p/v)) avant 5 h d'induction, et du TX100 (2% (v/v)) après 48 h d'induction, et suivi d'une incubation de 24 h à 30°C et à pH de 8,0. Aussi les profils d'activité enzymatique de la pullulanase recombinante en fonction du pH et de la température furent établis. La température et le pH optimaux de l'activité pullulanase étaient respectivement, 70°C et 6,0. La préparation enzymatique a maintenu 50% de son activité catalytique après une incubation à 70°C pendant plus de 120 min.

Mots clés :

Pullulanase, amylase, *Thermus thermophilus* HB8, *Pichia pastoris*, optimisation, sécrétion, Triton X-100, osmolytes naturels, thermostabilité.

Abstract

The need to develop thermostable amylases, principally pullulanases, has increased in recent decades due to the importance of these enzymes in several bio-industries. This research project is part of a major trend in industrial development of the extracellular production of thermoduric enzymes, with a focus on pullulanases.

A literature review on advances and challenges in the extracellular production of thermostable pullulanases was realised. The review describes the problematic of homologous and heterologous production of pullulanases. This problematic can be summarized by the fact, in many cases, that there is a difficulty of extracellular production of pullulanases with high enzymatic activity. Structural modification of these enzymes via different protein engineering techniques has allowed the development of highly thermostable pullullanases and/or having a remarkable catalytic activity. However, these techniques are expensive and require the development of more efficient bioinformatics tools that better control the modification process.

A study was conducted to determine the potential of *Thermus thermophilus* HB8 for accumulating a high level of extracellular, thermostable amylolytic enzymes, principally pullulanase. Initial production tests indicated clearly that only very low levels of amylolytic activity could be detected, solely from cells after extraction using the mild, non-ionic detergent Triton X-100 (TX100). A sequential optimization strategy, based on statistical designs, was used to enhance greatly the production of extracellular amylolytic activity in order to achieve industrially attractive enzyme titers. Focus was placed on the optimal level of initial biomass concentration, culture medium composition and temperature for maximizing extracellular amylolytic enzyme accumulation. Empirical models were then developed describing the effects of the experimental parameters and their interactions on extracellular amylolytic enzyme production. Following such efforts, extracellular amylolytic enzyme accumulation was increased more than 70-fold, with enzyme titers in the 76 U/mL range. The crude extracellular enzyme was thereafter partially characterized. The optimal temperature and pH values were found to be 80°C and 9.0, respectively. 100% of the initial enzyme activity could be recovered after incubation for 24 h at 80°C, therefore, proving the very high thermostability of the enzyme preparation.

A second study on the ability of *Pichia pastoris* to produce extracellularly pullulanase type 1 of *Thermus thermophilus* HB8 was conducted. The effects of different parameters on the extracellular production of the pullulanase were determined *i.e.* secretion signal peptide (SP) sequences, presence of TX100 at different concentrations and different addition times, presence of different natural osmolytes, pH and temperature. The production of extracellular pullulanase was increased more than 40-fold by adding K-glutamic acid (0.40% (w/v)), 5 h before the induction, and TX100 (2% (v/v)) after 48 h of induction, and followed by 24 h of incubation at 30°C and at pH 8.0. Also, the profiles of enzymatic activity as a function of pH and temperature were established. The optimal temperature and pH of the pullulanase activity were 70°C and 6.0, respectively. The enzyme preparation maintained 50% of its activity after incubation at 70°C for more than 120 min.

Keywords:

Pullulanase, amylase, *Thermus thermophilus* HB8, *Pichia pastoris*, optimization, secretion, Triton X-100, natural osmolytes, thermostability.

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Liste des abréviations

a.a : Acide aminé

ADN : Acide désoxyribonucléique

ARN : Acide ribonucléique

ATP : Adénosine triphosphate

dNTP : Désoxyribonucléide

ddNTP : Didésoxyribonucléide

bp : *Base pairs*

CD : Cyclodextrine

CDs : Cyclodextrines

CGTase : Cyclodextrines glycosyltransférases

Da : Dalton

DO : *Dissolved oxygen* (oxygène dissout)

EDTA : (Disodium) éthylènediamine tétraacétate

GH : Glycoside hydrolase

IPTG : Isopropyl p-D-thiogalactopyranoside

Kb : *Kilobase pairs*

kDa : Kilo Dalton

LB : Luria-Bertani

PAGE : *Polyacrylamide gel electrophoresis* (électrophorèse sur gel de polyacrylamide)

PS : Peptide signal

OD : *Optical density* (densité optique)

Tris : Tris-(hydroxyméthyl)-éthylamine

TX100 : Triton X-100

SDS : Sodium dodécyl sulphate

v/v : Volume par volume

w/v : Weight-by-volume (poids par volume)

Tableau 1: Les acides aminés et leurs codes

Code	Abréviation	Acide aminé	Code	Abréviation	Acide aminé
A	Ala	Alanine	N	Asn	Asparagine
C	Cys	Cystéine	O	Pyl	Pyrrolysine
D	Asp	Acide aspartique	P	Pro	Proline
E	Glu	Acide glutamique	Q	Gln	Glutamine
F	Phe	Phénylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Sérine
H	His	Histidine	T	Thr	Thréonine
I	Ile	Isoleucine	U	Sec	Sélénocystéine
K	Lys	Lysine	V	Val	Valine
L	Leu	Leucine	W	Trp	Tryptophane
M	Met	Méthionine	Y	Tyr	Tyrosine

Chapitre 1. Introduction

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1.1. Contexte du projet

Vu les fluctuations imprévisibles du prix du pétrole, et le besoin de diminuer les émissions de CO_2, il y a une forte demande pour le développement de produits exploitant et valorisant les ressources naturelles et renouvelables. La biotechnologie contribue grandement à la réalisation de ce grand objectif. En effet, la fabrication de biocarburants, de molécules plateformes et de matériaux biosourcés est devenue un marché mondial important dont les recettes pourraient atteindre 727.1 milliards de dollars américains en 2025 (Market research report, 2017).

Parmi les produits biochimiques qui ont eu une attention particulière à l'échelle industrielle, il y a les enzymes, lesquelles représentent un marché mondial de 2.2 milliards d'euros, dont 25% est constitué par des amylases (De Souza and Oliveira Magalhães, 2010). 70% de ce marché mondial de biocatalyseurs est dominé par la compagnie Novozymes et par sa rivale, la firme Diversa. La première utilisation industrielle d'une enzyme a eu lieu en 1915, dans le secteur des détergents (Fujiwara, 2002).

Actuellement, plus de 3000 enzymes ont été identifiées et caractérisées (Gupta *et al.*, 2014). Elles sont issues généralement de moisissures (champignons), de levures, et de bactéries mésophiles, dû aux avantages offerts par ces microorganismes, tels que la facilité et le contrôle de leur manipulation génétique, et la possibilité d'obtenir des enzymes avec des caractéristiques spécifiques désirées (De Souza and Oliveira Magalhães, 2010). La moitié des enzymes industrielles sont produites par des levures et des moisissures, 30% par la bactérie *E. coli*, 8% par des animaux et 4% par des plantes (Yang, 2013). Cependant, ces enzymes standards ne répondent pas à toutes les attentes industrielles, en raison de leur dénaturation dans les conditions souvent sévères utilisées dans les procédés de transformation/production (Gupta *et al.*, 2014).

Dans ce contexte, l'attention s'est dirigée vers les extrêmozymes, enzymes fonctionnelles à des conditions extrêmes, produites naturellement par des microorganismes extrêmophiles. Ces extrêmophiles sont des organismes, généralement des procaryotes (bactéries et archaea) ne pouvant survivre et croître que dans des habitats à conditions physiques et/ou géochimiques extrêmes telles que : acidité, alcalinité, haute pression, haute concentration en sels, et/ou hautes ou basses températures, et présence de produits toxiques dans leur

environnement (van den Burg, 2003). La plupart des composés cellulaires de ces microorganismes extrêmophiles (protéines, acides nucléiques et lipides) sont stables dans de telles conditions extrêmes de croissance (Elleuche *et al.*, 2014).

Dans la perspective d'exploiter les extrêmophiles en industrie, plusieurs programmes de recherche ont été lancés. Les plus remarqués sont ceux appuyés par l'Union Européenne depuis 1982 dont le thème était « *Extrêmophiles-cellules-usines* » avec un fonds de 20 millions de dollars pour une durée de 6 ans, et qui a formé 40 groupes de recherche académiques et industriels (Persidis, 1998). Aussi, d'autres programmes étaient lancés aux États-Unis et au Japon, auxquels se sont jointes plusieurs compagnies telles que : Amersham Pharmacia Biotech (Cleveland, OH), Archaenzyme Ltd. (Jerusalem, Israël), et Diversa (San Diego, Ca) (Persidis, 1998). Ces programmes ont contribué grandement à la caractérisation génomiques et protéomique de ces microorganismes.

En effet, le criblage de l'ADN de ces organismes a permis la découverte de nouvelles molécules ayant une haute valeur ajoutée et, par conséquent, la possibilité de leur introduction dans diverses applications industrielles, générant ainsi des bénéfices financiers significatifs. Néanmoins, ces études de criblage représentent seulement une première étape d'un processus qui doit aboutir à l'exploitation de ces enzymes en industrie.

Pour atteindre ce but, deux stratégies furent adoptées par les chercheurs. La première consiste en l'optimisation de la production de l'extrêmozyme par le microorganisme extrêmophile lui-même, par l'amélioration de la composition du milieu de culture et l'optimisation du mode de production/culture (*e.g. fed-batch*, recyclage des cellules, mode continu) (Ibrahim and Steinbüchel, 2010). Cette approche nécessite l'utilisation d'équipements adaptés pour la croissance cellulaire dans des conditions extrêmes (*e.g.* utilisation de bioréacteurs résistant à la corrosion, aux hautes températures et aux hautes pressions) (van den Burg, 2003). La deuxième stratégie est l'expression intensive d'enzymes recombinantes dans un hôte biologique facilement cultivable en industrie par l'exploitation des techniques du génie génétique. La plupart des protéines recombinantes dans le domaine pharmaceutique sont produites par *E. coli* (30%), des levures (20%) et par des eucaryotes avancés (cellules de mammifères ou d'insectes) (Yang, 2013).

Effectivement, le clonage d'un gène et son expression dans différents hôtes mésophiles est devenu un outil efficace et maîtrisé pour l'introduction de la majorité des extrêmozymes en industrie (Elleuche *et al.*, 2014). Cela est possible grâce à la disponibilité des séquences

génomiques complètes de plusieurs microorganismes, la possibilité de prévision théorique des fonctions des protéines en se basant sur les séquences nucléotidiques des gènes, l'existence d'algorithmes efficaces d'optimisation des séquences des gènes en fonction de l'hôte d'expression envisagé, et l'existence de techniques chimiques efficaces pour la synthèse d'ADN.

Parmi les procédés enzymatiques couvrant de larges domaines industriels, allant de l'alimentaire jusqu'aux détergents, ceux employant des amylases, enzymes de conversion de l'amidon, sont parmi les plus importants (Miller and Blum, 2010). La structure moléculaire de l'amidon est un paramètre majeur dans le choix des amylases pour sa conversion en sirop concentré de glucose, maltose, fructose, ou cyclodextrines (Martin and Smith, 1995). En effet, l'amidon, principale matière première de plusieurs industries, est un polymère d'unités de glucose liées par deux types de liaison glycosidique : la liaison α-1,4 et la liaison α-1,6 (van der Maarel *et al.*, 2002). Les types d'enzyme participant à l'hydrolyse de l'amidon lors de sa liquéfaction et de sa saccharification sont multiples. Ils peuvent être subdivisés en fonction de leur capacité d'hydrolyser les types de liaison glycosidique en trois groupes : 1/ les enzymes hydrolysant les liaisons α-D-1,4, telles les α- et β-amylases, 2/ les enzymes hydrolysant les liaisons α-D-1,6, telles les pullulanases type 1; et 3/ les enzymes hydrolysant en même temps les deux types de liaison glycosidique telles les glucoamylases et les amylopullulanases.

Les biocatalyseurs (enzymes) exploitables en amidonnerie industrielle sont les α-amylases, les β-amylases, les glucoamylases et les pullulanases (Plant *et al.*, 1986). Ces enzymes doivent être idéalement actives et stables à des températures supérieures à 60°C, à des valeurs de pH relativement faibles, allant de 4.5 à 6.0, pendant des durées allant de 24 à 72 h (Gomes *et al.*, 2003; Miller and Blum, 2010). Les enzymes amylolytiques des microorganismes thermophiles, en théorie, devraient bien répondre à de tels besoins, ce qui explique le lancement de plusieurs projets de recherche sur l'identification, la caractérisation et la production homologue ou hétérologue de ces thermozymes (Miller and Blum, 2010).

Les thermozymes, enzymes issues des thermophiles, sont caractérisées par une thermostabilité et une résistance à certains composés chimiques dénaturants pour les protéines, tels les surfactants et les solvants organiques qui sont souvent utilisés dans les procédés industriels traditionnels (Elleuche *et al.*, 2014). Les microorganismes thermophiles ont été isolés de différentes zones écologiques, comme les sources d'eaux chaudes et les mers profondes, mais aussi de zones industrielles chaudes telles que les centrales

géothermiques et les systèmes de traitement des eaux usées (Vieille and Zeikus, 2001). Ils sont classés en fonction de l'intervalle de températures pour leur croissance optimale ; les thermophiles modérés (50 à 60°C), les thermophiles extrêmes (60 à 80°C), et les hyperthermophiles (80 à 110°C). Les thermophiles bactériens extrêmes sont principalement distribués parmi les genres *Bacillus*, *Clostridium*, *Thermoanaerobacter*, *Thermus*, *Fervidobacterium*, *Rhodothermus*, *Thermotoga* et *Aquifex*. La plupart des hyperthermophiles sont des archées des genres *Pyrobaculum*, *Pyrodictium*, *Pyrococcus* et *Melanopyrus,* dont les températures optimales de croissance peuvent atteindre de 103°C à 110°C (Gupta *et al.*, 2014). L'activité optimale de la plupart des thermozymes est atteinte à des conditions proches ou même plus extrêmes que celles de la croissance de leur hôte biologique naturel (Vieille and Zeikus, 2001).

Des thermozymes recombinantes ont été introduites dans différents secteurs industriels (*e.g.* la biotransformation de l'amidon, la raffinerie, l'industrie pharmaceutique, etc.). Par exemple, la L-aminoacylase recombinante de *Thermococcus litoralis* est disponible commercialement pour la production de molécules optiquement pures, qui sont utilisées dans la formulation de médicaments. Pour un meilleur rendement de cette enzyme, elle est immobilisée par bioencapsulation, par absorption dans un support solide, ou par liaison covalente avec une matrice, telle que le Glyoxyl-Sepharose ou des monolithes (Satyanarayana *et al.*, 2013).

Il y a aussi la xylanase B recombinante tirée de l'hyperthermophile *Thermotoga maritima*, et la laccase recombinante de *Thermus thermophilus* exploitée dans l'industrie du papier et des fibres. Ces deux enzymes ont permis, respectivement, une diminution de l'utilisation du chlore et du peroxyde d'hydrogène dans les procédés industriels pertinents (Satyanarayana *et al.*, 2013).

Notre recherche s'inscrit donc dans ce contexte général, et elle s'intéresse particulièrement à la production d'amylases thermostables utilisables à l'échelle industrielle pour l'hydrolyse des liaisons glycosidiques de type α-1,4 et α-1,6 de l'amidon.

Les pullulanases sont des enzymes essentielles dans l'étape de saccharification de l'amidon. D'une part, elles hydrolysent les liaisons glycosidiques de type α-1,6 se trouvant aux points de branchement entre l'amylose et l'amylopectine, et d'autre part, elles facilitent le fonctionnement des β-amylases. La présence de pullulanases dans la saccharification permet

une augmentation de 20 à 25% de la production de glucose (Hii *et al.*, 2012b). Pour cette raison, une attention particulière fut dirigée vers la production de pullulanase thermostable.

1.2. Problématique du projet

La production de pullulanases microbiennes thermostables a généré un intérêt industriel ces dernières années. La diversité de ce type d'enzymes en fonction de la nature des substrats sur lesquels elles agissent, leurs mécanismes d'action et la nature des produits obtenus leurs donnent une importance croissante dans différentes industries (alimentaire, pharmaceutique et chimique) (Doman-Pytka and Bardowski, 2004). Elles sont utilisées conventionnellement dans les procédés d'hydrolyse de l'amidon, en combinaison avec les amyloglucosidases, les α- et β-amylases, pour la production des sirops à haute concentration en glucose et en maltose (Xu *et al.*, 2014). Aussi, ces biocatalyseurs sont exploités dans les procédés de gélatinisation de l'amidon pour l'augmentation de la production des cyclodextrines. D'autres pullulanases alcalines peuvent être exploitées comme additifs dans les détergents domestiques (Hii *et al.*, 2012b).

Malgré l'identification et la caractérisation de plusieurs types de pullulanases thermoduriques, leur intégration dans les applications industrielles reste encore très limitée. Actuellement, une seule pullulanase est disponible sur le marché pour une utilisation industrielle, et elle est commercialisée par la compagnie Novozymes. Il s'agit de la pullulanase de *Bacillus acidopullulyticus* exprimée par *B. subtilis*.

Les travaux de recherche focalisant sur l'optimisation de la production de ces enzymes utilisent soit l'approche directe d'optimisation de la croissance cellulaire des microorganismes extrêmophiles naturels, soit l'approche de la surexpression du gène d'intérêt dans des hôtes microbiens mésophiles. Par conséquent, plusieurs pullulanases thermostables ont été exprimée dans *Escherichia coli* (N. Ahmad *et al.*, 2014; Ben Messaoud *et al.*, 2002), et *Bacillus subtilis* (Su *et al.*, 2010). Néanmoins, il a été constaté que la plupart des pullulanases recombinantes sont intracellulaires, ou produites à faible taux au niveau extracellulaire (Doman-Pytka and Bardowski, 2004). Ce qui oblige une lyse cellulaire pour leur extraction, et par conséquent, la libération de plusieurs protéines, compliquant ainsi le processus de purification de l'enzyme cible.

Seulement trois efforts de recherche ont visé la production de pullulanases recombinantes dans la levure *Pichia pastoris,* malgré les divers avantages que présente cette levure, dont la facilité de sa culture à haute densité cellulaire, et sa capacité à produire de manière

extracellulaire la protéine hétérologue voulue (Akeboshi *et al.*, 2003; Xu *et al.*, 2006). L'un des rapports a montré que la séquence de sécrétion (SP) fusionnée au gène codant la pullulanase avait un effet significatif sur le type d'accumulation de celle-ci intra ou extracellulaire. Les trois recherches ont exploité les vecteurs pPIC9, pPIC9K, et pGAPZalpha produits par Invitrogen, dont le SP de facteur-alpha chez *Saccharomyces cerevisiae* et intégré. D'après la recherche bibliographique, seulement ces vecteurs d'expression ont été exploités visant la production extracellulaire de protéines recombinantes. Cependant, leur utilisation ne peut être que dans le cadre académique.

Les pullulanases produites par les thermophiles extrêmes sont caractérisées par une importante hydrophobicité, ce qui génère une grande thermostabilité de ces protéines (Farhat-Khemakhem *et al.*, 2013). Aussi, il y a celles qui présentent des domaines transmembranaires. Cela implique que plusieurs de ces pullulanases forment des agrégats au niveau intracellulaire (Duan *et al.*, 2015), ou sont ancrées dans la membrane cellulaire (Choi and Cha, 2015), ce qui empêche leur libération au niveau extracellulaire.

Thermus thermophilus HB8 est l'une des bactéries thermophiles extrêmes capables de produire des amylases (Fatoni and Zusfahair, 2012; Shaw *et al.*, 1995). Cependant, aucun travail de recherche n'a essayé ni d'optimiser la production de ces amylases par voie homologue ou hétérologue, ni de déterminer leurs caractéristiques physico-chimiques. Il y a une seule étude qui a pu démontrer que cette bactérie est capable de produire une pullulanase d'un maximum de 0.016 U/mL d'enzyme semi-purifiée, à 70°C et à un pH 6.0.

Malgré l'expression du gène de la pullulanase de *T. thermophilus* HB8 par *E. coli*, le niveau de production était faible, et l'accumulation de la protéine était au niveau intracellulaire (Tomiyasu *et al.*, 2001). **Cela peut être expliqué par trois hypothèses que nous avons proposées : 1/ absence de la séquence de sécrétion qui peut faciliter la sécrétion de l'enzyme, 2/ *E. coli* n'est pas capable de replier correctement la protéine ce qui peut former des corps d'inclusion, et 3/ formation d'agglomérat de pullulanase au niveau intracellulaire dû à sa structure moléculaire.**

Dans le cadre de ce contexte spécifique, quatre questions ont été posées :

1. Est-ce possible d'optimiser la production d'enzymes amylolytiques, principalement la pullulanase, directement par *Thermus thermophilus* HB8 sans passer par la production recombinante ?

2. Est-ce que *Pichia pastoris* est capable de produire de façon extracellulaire la pullulanase de *Thermus thermophilus* HB8?

3. Quel est l'effet des séquences peptidiques-signal sur la production extracellulaire de la pullulanase par *Pichia pastoris* ?

4. Quelles sont les paramètres critiques qui permettent une amélioration de la production extracellulaire de la pullulanase de *Thermus thermophilus* HB8 par *Pichia pastoris* ?

1.3. Objectifs du projet

L'objectif principal de cette étude est la génération d'une preuve de concept visant la production extracellulaire d'une pullulanase thermodurique de *T. thermophilus* HB8 par un procédé de fermentation pouvant être exploité à l'échelle industrielle.

Deux hypothèses de recherches ont été formulées :

1- **Par l'optimisation statistique du milieu de culture de *T. thermophilus* HB8, la bactérie peut être un producteur industriel efficace d'amylases, principalement de pullulanase.**
2- **Par l'exploitation de *P. pastoris*, la pullulanase de *T. thermophilus* HB8 peut être produite de façon extracellulaire.**

Cinq (05) objectifs spécifiques ont été définis pour vérifier ces deux hypothèses et répondre aux questions précitées :

1/ Élaboration d'une revue de littérature sur les avancées technologiques développées jusqu'à présent pour la production extracellulaire de pullulanases thermoduriques, et les différentes problématiques rencontrées qui limitent l'utilisation de cette enzymes au niveau industriel ;

2/ Optimisation de la production d'enzymes amylolytiques, principalement la pullulanase type 1 , par *Thermus thermophilus* HB8 en évaluant l'effet de différents facteurs environnementaux sur l'activité amylolytique produite, exploitant des techniques statistiques à multi-variables ;

3/ Expression du gène de la pullulanase type 1 de *Thermus thermophilus* HB8 par *Pichia pastoris*, en évaluant l'effet de six (06) séquences peptides-signal sur la production extracellulaire de l'enzyme. Ces peptides-signal se trouvent dans un vecteur d'expression

pD912, exploité pour la première fois pour la production de pullulanase, et les souches générées peuvent être transférées directement pour la production au niveau industriel ;

4/ Identification de paramètres environnementaux permettant l'amélioration de la production extracellulaire de la pullulanase par *P. pastoris*;

5/ Comparaison des propriétés catalytiques de la pullulanase recombinante produite par *P. pastoris* avec celles produite par la souche d'origine et par *E. coli*.

1.4. Démarche du projet de recherche et organisation générale du document

Le but principal de la thèse consistait en la génération d'une preuve technique de concept visant la production extracellulaire d'une pullulanase thermodurique de *T. thermophilus* HB8.

Premièrement, une revue de littérature fut réalisée (**chapitre 2**), composée de deux parties : la **partie 1** présente les différents types d'amylases et leurs caractéristiques, et la **partie 2** (**article 1**) présente les avancées technologiques ainsi que les problématiques rencontrées pour la production extracellulaire de pullulanases thermostables. Cette revue a permis la compréhension des aspects critiques qui ont un grand impact sur la réussite d'un procédé de production extracellulaire d'une biomolécule en général, et d'une pullulanase thermostable en particulier.

Deuxièmement, une étude expérimentale fut conduite pour évaluer la faisabilité de produire de façon extracellulaire des amylases, en particulier des pullulanases directement de *T. thermophilus* HB8 (**chapitre 3 : article 2**). Le point de départ était la conduite des cultures cellulaires de *T. thermophilus* HB8 pour confirmer que ce thermophile est capable de produire des amylases, en particulier des pullulanases. Effectivement, il a était observé que *T. thermophilus* HB8 est capable de produire des amylases ancrées à la surface cellulaire, mais aucune activité de pullulanase ne fut vraiment détectée. Le fait que le « cocktail » d'amylases produit avait des propriétés très intéressantes, principalement une haute thermostabilité, nous avons entrepris une démarche d'optimisation en utilisant des méthodes statistiques subséquentes pour maximiser l'activité enzymatique produite. Du fait que la littérature confirme que *T. thermophilus* HB8 est capable de produire une pullulanase type 1 extracellulaire, une hypothèse a été émise *i.e.* par l'optimisation de l'activité amylolytique produite par ce microorganisme, le niveau de production de pullullanase peut augmenter aussi.

La méthode du plan factoriel partiel fut utilisée pour effectuer un criblage de six (06) paramètres liés aux conditions environnementales ayant un effet significatif sur la production d'activité amylolytique produite par *T. thermophilus* HB8. Les facteurs sélectionnés furent optimisés en utilisant un plan composite central (*Central composite design*). Le niveau de production de cette dernière fut augmenté plus de 70 fois par une optimisation statistique des conditions environnementales, et l'activité enzymatiques désirée (amylase) fut retrouvée dans le surnageant, sans utiliser de surfactant pour libérer ces enzymes. Cependant, aucune activité pullulanase ne fut détectée ni avant, ni après ce processus d'optimisation. Cela peut être expliqué en partie par le fait qu'aucune méthode de concentration et de purification ne fut effectuée lors cette étude.

Les propriétés du « cocktail » d'amylases produit furent déterminées, à savoir les profils d'activité amylolytique en fonction du pH et de la température.

Troisièmement, une étude de production recombinante de la pullulanase type 1 de *T. thermophilus* HB8 par *P. pastoris* fut éffectuée (**chapitre 4 : article 3**). Le point de départ de l'étude fut une évaluation de l'effet de six séquences de sécrétion sur l'expression de la pullulanase recombinante par *P. pastoris*. Pour la première fois, le vecteur pD912 fut exploité pour la production de la pullulanase de *T. thermophilus* HB8 par *P. pastoris*.

Il a été observé une faible production extracellulaire de cette enzyme, malgré l'utilisation de la séquence de sécrétion du facteur alpha de *S. cereviciae*, lequel a été utilisée dans de nombreuses études, et qui a démontré sa capacité de faciliter la sécrétion de la protéine cible. Pour vérifier que la structure du vecteur pD912 n'a pas eu d'effet sur la production intracellulaire de la protéine, les vecteurs pPIC9K et pGAPZalpha, connus par leur production extracellulaire furent aussi testés. Seulement une accumulation intracellulaire de la pullulanase fut obtenue.

L'étude s'est poursuivie par l'identification de facteurs environnementaux qui peuvent faciliter la libération/l'extraction de la protéine dans le milieu de culture sans passer par une lyse cellulaire. En effet, pour la première fois une évaluation de l'effet du Triton X-100 sur l'accumulation extracellulaire d'une pullulanase par *P. pastoris* fut réalisée. Le niveau de concentration et le temps d'ajout du Triton X-100 furent testés. Aussi les effets d'autres paramètres (osmolytes naturels, pH, température et source de carbone) furent aussi évalués. Cette étude présente une nouvelle méthode d'extraction chimique de la pullulanase recombinante en cours du procédé de fermentation avec *P. pastoris*, qui peut être utilisée

pour la récupération d'autres protéines produites au niveau intracellulaire. L'étude fut complétée par la détermination des propriétés de base de la pullulanase recombinante produite, à savoir les profils d'activité pullulanase en fonction du pH et de la température. La thermostabilité de la protéine, et l'effet de plusieurs ions et du Triton X-100, à différentes concentrations, sur l'activité enzymatique furent aussi évalués.

Finalement, à partir de ces travaux de recherche, des conclusions et des recommandations sont présentées dans le **chapitre 5**

Chapitre 2 : État de l’art

Chapitre 2. État de l'art

Partie 1. Amylases : Enzymes de conversion de l'amidon

La structure moléculaire de l'amidon est un paramètre majeur dans le choix des enzymes pour son hydrolyse (Martin and Smith, 1995). Effectivement, les amylases sont subdivisées en quatre types en fonction de leurs mécanismes d'action sur l'amidon : endo-amylases, exo-amylases, transférases et enzymes débranchantes (Hii *et al.*, 2012b). Ces enzymes sont groupées dans plus de 100 familles de glycoside hydrolases (GH) en se basant sur la similarité des structures et des séquences (http://www.cazy.org/) (Han *et al.*, 2013).

2.1.1. Classification des amylases

2.1.1.1. Endo-amylases

Les α-amylases, enzymes les plus connues de ce type, sont capables de rompre de façon aléatoire les liaisons glycosidiques α-1,4 de la partie intérieure de l'amylose et de l'amylopectine, pour donner des oligosaccharides linéaires et ramifiés de différentes longueurs (van der Maarel *et al.*, 2002). Les α-amylases ne sont pas identiques et produisent différents oligosaccharides dépendamment de leur site actif ainsi que du substrat utilisé (amylose, amylopectine, glycogène, dextrines, etc.). Elles sont caractérisées par deux propriétés qui sont, d'une part, des produits finaux tous de la configuration alpha et, d'autre part, leur mécanisme réactionnel est de type endo, c'est-à-dire, leur action s'effectue à l'intérieur des chaines d'amylose et d'amylopectine (Satyanarayana *et al.*, 2013).

Une α-amylase idéale pour la liquéfaction de l'amidon doit avoir une activité optimale à 100°C à pH 4.0 à 5.0, et sa stabilité doit être indépendante de la présence d'ions Ca^{2+} (Vieille and Zeikus, 2001). Parmi les α-amylases thermostables identifiées et qui ont montré une activité maximale à haute température, il y a celles du genre *Pyrococcus* et de *Methanococcus jannaschii* dont les températures d'activité optimale sont, respectivement, 100°C et 120°C (Satyanarayana *et al.*, 2013). Aussi il y a celle isolée d'un réacteur de biométhanisation et montrant une activité spécifique maximale de 1000 U/mg de protéines à 80°C, et une stabilité de 3 h à 70°C (Elleuche *et al.*, 2014). Les amylases thermostables utilisées dans l'industrie de l'amidon sont celles de *Bacillus stearothermophilus* produites par *Bacillus licheniformis* (Gomes *et al.*, 2003).

2.1.1.2. Exo-amylases

Ce type d'enzyme coupe les liaisons α-1,4 seulement, dans le cas des β-amylases, ou les deux types de liaison, α-1,4 et α-1,6, dans le cas des amyloglucosidases (ou glucoamylases) et des α-glucosidases (van der Maarel *et al.*, 2002). Leur mécanisme d'action est de type externe, c'est-à dire, une action au niveau du glucose résiduel non-réducteur externe de la chaine et, donc, le seul produit final est le glucose, à l'exception des β-amylases qui peuvent rompre les liaisons de type α-1,4 pour donner du maltose et des β-dextrines (Hii *et al.*, 2012b). Les β-amylases et les amyloglucosidases convertissent la configuration anomérique alpha en configuration beta. La plupart des β-amylases et des glucoamylases utilisées dans l'industrie sont d'origine végétale et fongique, respectivement (Satyanarayana *et al.*, 2013). Une β-amylase idéale pour la saccharification de l'amidon (pH 4.5, stable de 48 à 72 h) doit être active à pH faible et être caractérisée par une vitesse de réaction importante, de telle sorte qu'elle permettra une réduction du temps opératoire (Vieille and Zeikus, 2001).

2.1.1.3. Transférases

Les transférases sont des enzymes capables de rompre les liaisons de type α-1,4 de la molécule-donatrice et de transférer la partie-donatrice à un accepteur glycosidique, pour la formation de nouvelles liaisons glycosidiques (Hii *et al.*, 2012b). Parmi ces enzymes, il y a les amylomaltases et les cyclodextrines glycosyltransférases (CGTase), qui forment des liaisons glycosidiques de type α-1,4, et des enzymes de branchement, qui forment des liaisons de type α-1,6 (van der Maarel *et al.*, 2002). Les CGTase ont une exo-activité et une activité de transglycosylation qui permettent la cyclisation des oligosaccharides de 6, 7 ou 8 unités de glucose, pour donner, respectivement, des α-, β- et γ-cyclodextrines (CDs). Cela s'effectue, premièrement, par la rupture de liaisons α-1,4 et, deuxièmement, par la liaison entre les parties réduites avec les parties non-réduites. Les propriétés des CDs, telles que leur capacité d'encapsuler des molécules hydrophobes, leur permettent d'être utilisées pour différentes applications dans les industries alimentaires, cosmétiques et pharmaceutiques (Vieille and Zeikus, 2001).

2.1.4. Enzymes débranchant l'amidon

Ces enzymes catalysent la rupture des liaisons α-1,6 de l'amylopectine ou du glycogène. Selon Hii *et al.,* (2012b), elles sont subdivisées en deux groupes majeurs en fonction du mécanisme de débranchement, direct ou indirect.

a/ Débranchement indirect : ces enzymes sont appelées amylo-1,6-glucosidases, excrétées généralement par les animaux et les levures (Hii *et al.*, 2012b). Leur action s'amorce seulement quand la structure du substrat contient une unité de glucose liée par liaison α-1,6, ce qui nécessite l'intervention d'autres enzymes en amont, en premier lieu les phosphorylases (Cori and Larner, 1951), ensuite, les transférases (Nelson and Larner, 1970).

b/ Débranchement direct : ces enzymes sont subdivisées en deux classes, les isoamylases et les pullulanases. Ces deux classes hydrolysent des oligo- et poly-saccharides ayant des liaisons glycosidiques α-1,6. La différence entre les deux classes est que les isoamylases ont une action principalement sur le glycogène (polysaccharide ramifié de source animale) tandis que les pullulanases ont une action sur le pullulane (Hii *et al.*, 2012b). À noter que le pullulane est un polysaccharide d'origine fongique (*Aureobasidium pullulans)*, et est un α-glucane constitué d'unités de maltotriose liées par des liaisons glycosidiques α-1,6 (N. Ahmad *et al.*, 2014).

2.1.2. Pullulanases

Les pullulanases, enzymes appelées aussi α-dextrine 6-glucanohydrolases, pullulane 6-glucanohydrolases, limite-dextrinases ou amylopectine 6-glucanohydrolases, hydrolysent le pullulane (Matzke *et al.*, 2000; Xu *et al.*, 2014). Elles sont subdivisées en cinq types en fonction des substrats et des produits résultants : pullulanases type I, amylopullulanases, néopullulanases, isopullulanases, et pullulane hydrolases type III (Hii *et al.*, 2012b). Généralement, ces enzymes appartiennent à la famille des glycosides hydrolases 13 (GH13). Cependant, il existe six amylopullulanases décrites jusqu'à présent appartenant à la famille GH57 (Xu *et al.*, 2014).

La caractéristique majeure des pullulanases est le fait que ces enzymes n'agissent que sur des oligo- et poly-saccharides contenant des chaines avec au moins deux unités de glucose liées par des liaisons α-1,4, et ces chaînes sont liées par des liaisons α-1,6 (Hii *et al.*, 2012b). Cette spécificité de reconnaissance de la liaison α-1,6 permet aux pullulanases d'être exploitées dans les études de structure des oligo- et poly-saccharides) (Xu *et al.*, 2014).

2.1.2.1. Pullulanases type I

Ce type de pullulanases catalyse l'hydrolyse de façon préférentielle d'oligo- et poly-saccharides et de pullulane pour produire, respectivement, des α-1,4 oligomères linéaires et du maltotriose (Bertoldo *et al.*, 2004). Son mécanisme d'action est de type endo (Domań-Pytka and Bardowski, 2004). Plusieurs pullulanases de type I thermostables ont été

caractérisées à partir *d'Anaerobranca gottschalkii*, *Bacillus acidopullulyticus, Bacillus flavocaldarius, Caldicellulosiruptor saccharolyticus, Fervidobacterium pennivorans, Geobacillus stearothermophilus, Geobacillus thermoleovorans, Thermotoga maritima, Thermotoga neapolitana, Thermus aquaticus, Thermus caldophilus, et Thermus thermophilus* (Jasilionis *et al.*, 2014). La pullulanase utilisée dans l'industrie de glucose est celle de *Bacillus acidopullulyticus* produite par *Bacillus subtilis* par la compagnie Novozymes, ayant une activité optimale à 60°C et un pH optimal de 5.0 (Domań-Pytka and Bardowski, 2004).

2.1.2.2. Pullulanases type II (amylopullulanases)

Les amylopullulanases présentent un grand intérêt dans l'industrie de l'amidon, dû à leur capacité d'hydrolyser les deux types de liaisons présentes, α-1,6 et α-1,4, c'est-à-dire, elles possèdent une activité amylase et une activité pullulanase en même temps (Domań-Pytka and Bardowski, 2004). Elles permettent la production du maltotriose à partir du pullulane, et un mélange de produits composé majoritairement de glucose, maltose et maltotriose dans le cas des oligo- et poly-saccharides (Hii *et al.*, 2012b). Ce type de pullulanase est distribué dans les deux familles GH13 et GH57 (Xu *et al.*, 2014). Les amylopullulanases thermostables sont des candidates potentielles pour le remplacement des α- et β-amylases pour l'hydrolyse de l'amidon en une seule étape (liquéfaction et saccharification en même temps) (Nisha and Satyanarayana, 2013a). Principalement, elles sont thermostables et leur activité est indépendante de la présence d'ions Ca^{2+}. Cependant, leur faible taux d'hydrolyse de l'amidon est un facteur limitant leur utilisation en industrie. Par exemple, la α-amylase de *Pyrococcus furiosus* est active 44 fois plus que l'amylopullulanase provenant du même microorganisme (Vieille and Zeikus, 2001). Il existe des amylopullulanases capables de dégrader les cyclodextrines, par contre, d'autres sont inhibées par la présence de ces molécules (X. Li *et al.*, 2013).

2.1.2.3. Autres types de pullulanases

Il existe trois autres types de pullulanases mais qui ont été moins étudiées : les pullulane-hydrolases type I (néopullulanases), les pullulane-hydrolases type II (isopullulanases), et les pullulane-hydrolases type III.

Les néopullulanases et les isopullulanases n'ont pratiquement pas d'activité sur l'amidon, et elles peuvent exclusivement hydrolyser les liaisons α-1,4 du pullulane pour libérer respectivement le panose et l'isopanose (Hii *et al.*, 2012b). Elles sont hautement actives sur

les cyclodextrines (Nisha and Satyanarayana, 2013a). Des auteurs affirment que les néopullulanases ont une activité hydrolytique sur les liaisons α-1,4 et α-1,6, et une activité transglycosydique sur les deux types de liaisons glycosidique (Takata *et al.*, 1992).

Concernant les pullulane-hydrolases type III, ces enzymes hydrolysent les deux types de liaison glycosidique, α-1,4 et α-1,6, du pullulane pour donner un mélange composé majoritairement de panose, maltose et maltotriose, et dans le cas de l'amidon, les produits seront un mélange composé principalement de maltotriose et de maltose (Niehaus *et al.*, 2000).

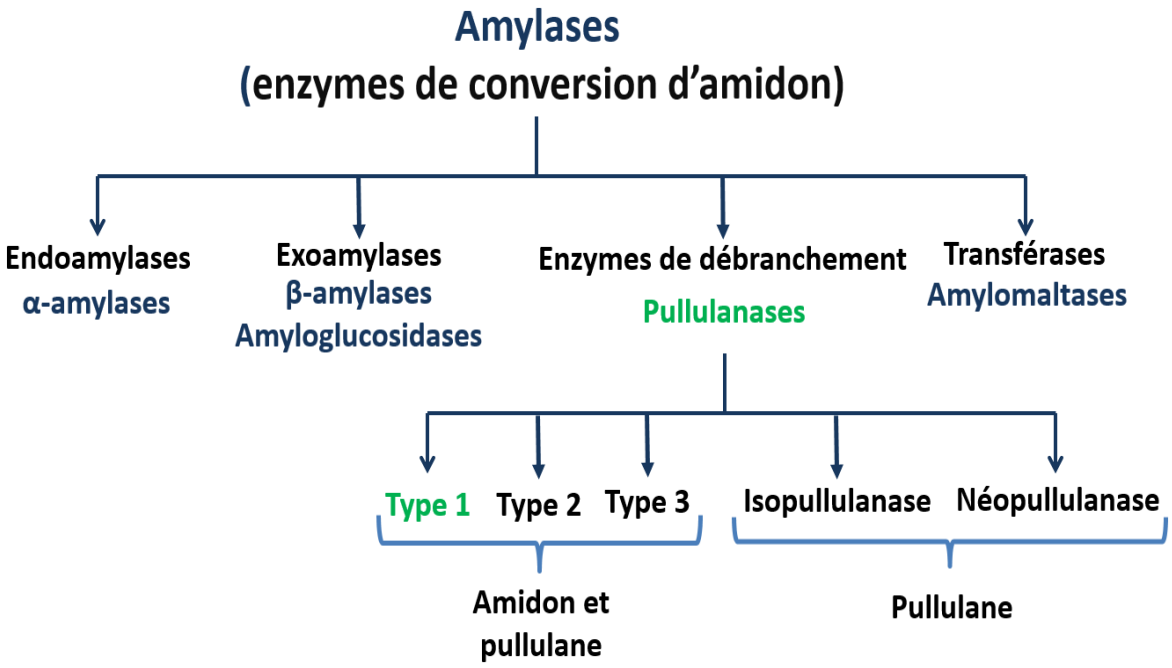

Figure 2.1 : Classification des amylases.

Partie 2. Production extracellulaire de pullulanases thermostables

Avant-propos

Titre de l'article

Avancés et défis à relever dans la production extracellulaire de pullulanases thermoduriques par leurs microorganismes d'origine et par des microorganismes recombinants : revue de littérature.

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L'article présenté dans ce chapitre concerne le premier objectif du projet, à savoir une revue de littérature critique qui permet d'exposer les avancées technologiques développés jusqu'à présent, ainsi que la compréhension des différentes problématiques rencontrées lors de la production extracellulaire de pullulanases thermoduriques. Cette revue de littérature permet de déterminer les aspects critiques qu'il faut considérer pour la réussite de la production extracellulaire de pullulanase pour des fins industrielles.

Résumé

Les pullulanases thermoduriques sont des enzymes débranchant l'amidon, nécessaires dans de nombreuses applications industrielles, principalement dans la production de sirops concentrés en glucose, en maltose et en fructose. À ce jour, une seule pullulanase, celle de *Bacillus acidopullulyticus*, est disponible sur le marché à des fins industrielles. Cette revue est une étude des avancées technologiques, ainsi que des défis majeurs rencontrés dans l'optimisation de la production extracellulaire de pullulanases thermoduriques, soit par leurs hôtes d'origine, soit par des hôtes recombinants. Les aspects critiques qui doivent être considérés lors de la production industrielle de pullulanase sont mis en évidence, principalement les paramètres influençant la solubilité, la thermostabilité et l'efficacité catalytique de l'enzyme. Cette revue fournit de nouvelles perspectives pour améliorer la

production extracellulaire de pullulanases thermoduriques, qui pourraient être aussi utilisées comme source d'inspiration pour l'amélioration de la production d'autres enzymes sur le plan industriel.

Mots clés

Pullulanase, expression homologue et hétérologue, génie des protéines, thermostabilité, solubilité, optimisation

Entête du manuscrit 1

Title

Advances and challenges in the production of extracellular thermoduric pullulanases by wild-type and recombinant microorganisms: a review

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Abstract

Thermoduric pullulanases, acting as starch-debranching enzymes, are required in many industrial applications, mainly in the production of concentrated glucose, maltose and fructose syrups. To date, just one pullulanase, that from *Bacillus acidopullulyticus,* is available on the market for industrial purposes. This review is an investigation of the major advances as well as of the major challenges being faced in the optimization of the production of extracellular thermoduric pullulanases either by their original hosts or by recombinant ones. The critical aspects linked to industrial pullulanase production, which should always be considered, are being emphasized, mainly those parameters influencing solubility, thermostability and catalytic efficiency of the enzyme. This review provides new insights for improving the production of extracellular thermoduric pullulanases in the hope that some of the new information could be applied to other industrially relevant enzymes.

Key words

Pullulanase, homologous and heterologous expression, protein engineering, thermostability, solubility, optimization.

2.2.1. Introduction

The enzyme pullulanase, also called α-dextrin endo-1,6-glucanohydrolase, pullulan-1,6-glucanohydrolase, limit-dextrinase or amylopectin 6-glucanohydrolase, *via* its direct debranching activity, catalyzes the hydrolysis of oligo- and polysaccharides composed of chains of glucose units linked by α-D-1,4 glucosidic bonds (at least two bonds), and these chains are linked by α-D-1,6 glycosidic bonds (Hii *et al.*, 2012b; Xu *et al.*, 2014). Due to its affinity for the α-D-1,6 glycosidic linkage, the pullulanase enzyme is used in the production of high-glucose, maltose and fructose syrups and in the production of bioethanol from starch (Duan *et al.*, 2013a). All these concentrated syrups find applications in the food, beverage and pharmaceutical industries. Moreover, a pullulanase enzyme may also be combined with proteases, lipases, β-glucanases, and xylanases in beer brewing (van Donkelaar *et al.*, 2016), and in detergent formulations for removing starch-based stains (Van Ee *et al.*, 1997). Pullulanases are gaining increasing interest in other biotechnological industries and serve as useful tools for structural studies of carbohydrates (Kunamneni and Singh, 2006), principally those of various starches (Hii *et al.*, 2012a). Furthermore, pullulanases could modify the molecular structure of the starch, called debranched starch, which has remarkable new properties and functionalities that meet the requirements of specific applications. Recently, debranched starch was used as a fat/protein replacer in food products, and control drug release as a tableting excipient in the pharmaceutical industry (Liu *et al.*, 2017).

Starch transformation is today an enzymatic process involving basically two main steps: liquefaction and saccharification (Figure 2.2) (Hii *et al.*, 2012b; Robyt, 2009; De Souza and Oliveira Magalhães, 2010). The saccharification of starch is a critical enzymatic step, usually involving β-amylase, pullulanase, and glucoamylase enzymes, and is carried out at temperatures above 60°C and at pH 4.5 for a duration from 24 to 72 hours (Chen *et al.*, 2015). This application naturally increases the demand for the development of thermoduric and acidoduric enzymes with high reaction rates and high half-life times. Starch transformation is preferably conducted at elevated temperatures, due mainly to decreased microbial and viral contamination risks, increased solubility of starch, decreased viscosity of the reaction mixture, shorter reaction times, all of these advantages leading to more economical processes (Ramesh *et al.*, 2001).

Several thermoduric pullulanases have been identified and characterized, originating from thermophilic and extreme thermophilic microorganisms. The production of some of these pullulanases has been optimized, using either the original host or a recombinant host (Table

2.1) (McCleary *et al.*, 2014). However, most of these thermoduric enzymes have not been produced at large scale for industrial purposes. To date, pullulanases are classified into five groups based on substrate preference and reaction products: pullulanases type 1, amylopullulanases, neopullulanases, isopululanases and pullulan hydrolases type III (Hii *et al.*, 2012b). According to the CaZy database (http://www.cazy.org), pullulanases belong to glycosidic hydrolases family 13 (GH13), except for six amylopullulanases that belong to the GH57 family (Chen *et al.*, 2013).

Considering the diversity of pullulanases and their burgeoning applications in environment-friendly processes, there remains a need to study these enzymes even more and to overcome their limitations. Many strategies have been proposed to increase production yields, thermostability and catalytic activity, either by screening wild-type microbial strains from Nature, by improving recombinant gene expression, or by protein engineering (Chen *et al.*, 2015). It is important to notice that extracellular production of enzymes provides many advantages in comparison to their accumulation in the cytoplasm. A direct consequence is simplicity of the enzyme recovery process. Increasing our understanding of the problems associated with the production of extracellular, thermostable pullulanases will lead undoubtedly to significant progress.

In this context, this review is providing an overview of the recent advances in extracellular thermoduric pullulanase production. The main focus will be on the critical aspects and important parameters that influence the level of extracellular production/accumulation of thermoduric pullulanases whatever the mode of production used *i.e.* wild-type microorganisms or recombinant production *via* microbial expression systems. In addition, parameters associated with the molecular structure of pullulanases that may improve thermostability, solubility, and catalytic efficiency of these enzymes will also be covered.

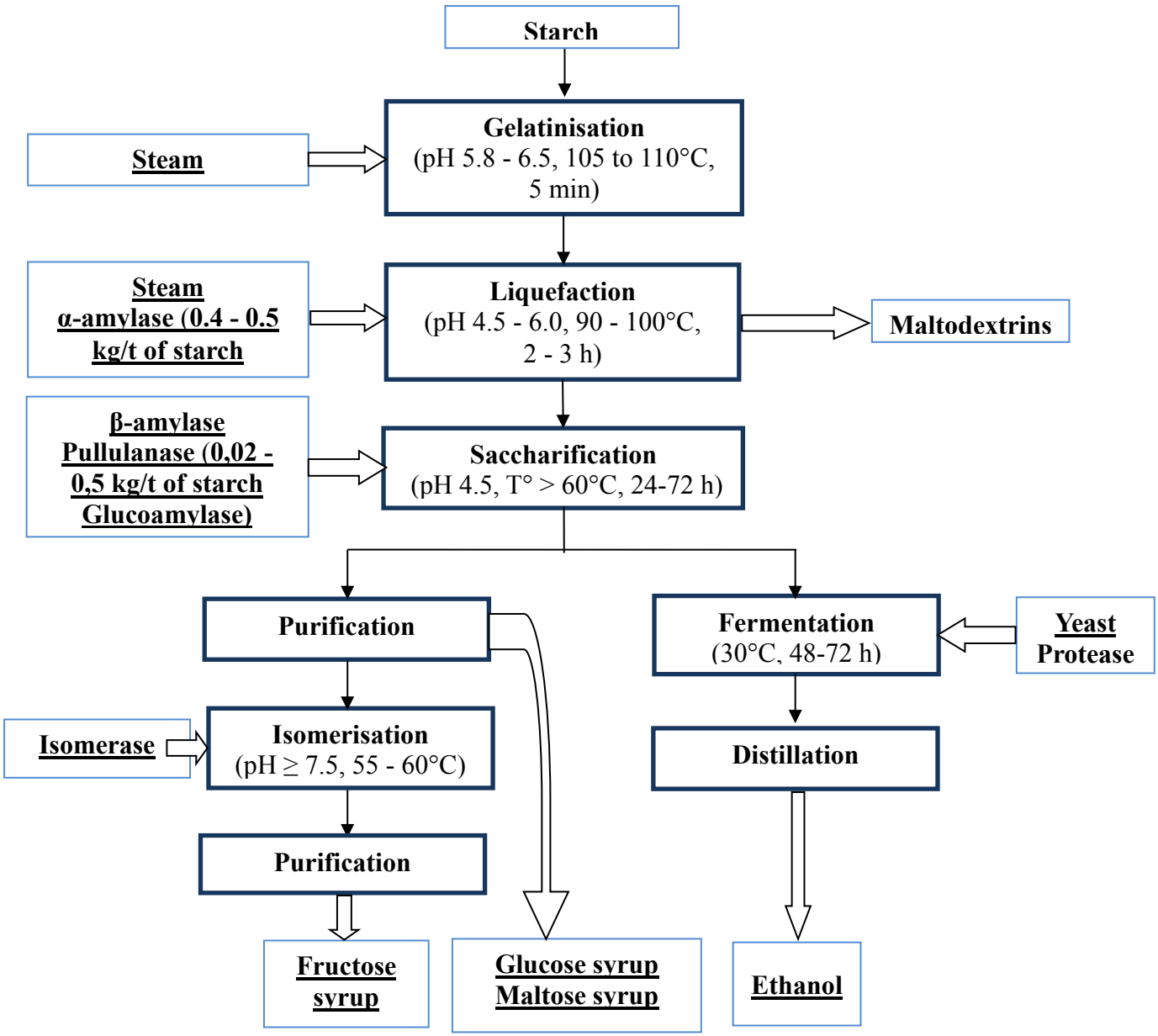

Figure 2.2: Industrial enzymatic processing of starch.

Table 2.1: Characteristics of some thermoduric pullulanases, their original host, and conditions for recombinant production (whenever applicable).

Expression host	Type of secretion	Expression vector	Optimal culture medium	Optimal operational conditions	Recovery method(s)	Optimal conditions for activity	Maximal enzymatic activity	Ref.
Bacillus acidopullulyticus **(101 kDa, 921 aa)**								
E. coli	Extracellular and intracellular	T7Lac operator and *lacI* gene	Three phases: TB in batch phase, 6.0 ml/l/h of glycerol in fed- batch phase and 0.1mM IPTG in induction phase	Three phases: 30°C in batch phase, 30°C to 20°C (3 h) in fed-batch phase and 16°C (28 h) in induction phase	-	60°C, pH 5.0	Soluble fraction: 1,156.32 U/mL	(Chen *et al.*, 2014)
Thermus thermophilus **HB27 (52 kDa, 475 aa)**								
E. coli	Extracellular and intracellular	Hsh promoter without putative signal peptide	LB	30°C, and induction (6h) by increasing the temperature to 42°C,	Cell lysis by sonication, heat treatment, and Ni–NTA affinity chromatography	70°C, pH 6.5 Activators: Mn^{2+} (5 mM), Fe2+, EDTA	Soluble fraction: 13.8 U/mL; insoluble fraction: 19.5 U/mg of proteins in 3.8 g of cells and 280U/mg of purified protein	(Wu *et al.*, 2014)
E. coli	Extracellular and intracellular	T7 promoter without signal peptide	LB, induction: 1 mM IPTG (4-10 h)	30°C	-		Soluble fraction: 3.8 U/mL	
Bacillus thermoleovorans **(80 kDa, 718 aa)**								
E. coli	Extracellular and intracellular	Trc promoter and amylase SP of *B. stearothermophilus*	LB, induction with 1mM IPTG	37°C, 10 h	-	75°C, pH 5.6–6.0, 0.1 mM Ca^{2+} improves thermostability	0.45 U/mL of supernatant fluid, and 2.6 U/mL of periplasmic fraction	(Zouari Ayadi *et al.*, 2008)

***Caldicellulosiruptor saccharolytics* (95 kDa, 825 aa)**								
E. coli	Extracellular	pNZ1037	LB	37°C, 16 h	-	85°C	-	(Albertson *et al.*, 1997)
***Fervidobacterium pennavorans* (93 kDa, 849aa)**								
E. coli	Intracellular	pSE420, with Trc	LB, 1 mM IPTG	37°C, 18 h	Heat treatment, anionic chromatography, dialysis, and ultrafiltration	80°C, pH 6 Inhibitors: $Zn2^{+}$, $Cu2^{+}$, and $Fe2^{+}$, α-, β et γ-cyclodextrins	0.43 U/mg in supernatant, and 75 U/mg of proteins in purified enzyme	(Bertoldo *et al.*, 1999)
Bacillus deramificans* (*101 kDa, 928 aa)								
E. coli	Extracellular and intracellular	pET20b(+) with T7 promoter and OmpASP	TB, 2.3 g/L betaine, induction at 15 g/L DCW, 0.4 g L^{-1} h^{-1} of lactose and l% of glycine	Growth phase: 30°C, induction phase: 25°C, 74 h	-	60°C, pH 4.5	1567.9 U/mL in the supernatant fluid	(Zou *et al.*, 2014)
Anaerobranca gottschalkii* (*96 kDa, 865 aa)								
E. coli	Intracellular	pETBlue-1 with T7 and the original SP	LB, 1 mM IPTG	37°C, 18 h	Cell lysis by sonication, heat treatment, anionic chromatography, dialysis, and ultrafiltration	70°C, pH 8.0 – 8.5, Inhibitors: Co^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+}, α, β et γ-cyclodextrins	11 U/mg of proteins in the cells and 56 U/mg of proteins	(Bertoldo *et al.*, 2004)

Staphylothermus marinus* (*75 kDa, 639 aa)								
E. coli	Intracellular	pTKNd6xH with BLMA promoter	LB	37°C, 20 h	Cell lysis, thermal treatment, chromatography, and dialysis	105°C, pH 5, 5 mM $CaCl_2$ Inhibitors: Zn2+, Mn^{2+}, Al^{3+}, Fe^{2+}, and Co^{2+}	42.1 U/mg of purified enzyme	(Li X. *et al.*, 2013)
***Thermococcus kodokarensis* (86 kDa, 765 aa)**								
E. coli	Extracellular	pET28 with T7 and the original SP, fusion Histidine tag	LB, 1 mM IPTG	37°C, 4 h	Affinity chromatography	100°C, pH 5.5-6.0, Inhibitors: Co2+, Mn^{2+}, Zn^{2+}, EDTA, Cu^{2+} and Fe^{2+}	118.36 U/mg of proteins	(Han *et al.*, 2013)
***Bacillus naganoensis* (101 kDa, 926 aa)**								
B. subtilis	Extracellular and intracellular	W600 strain, pMA0911 with P_{43} and LipA SP	LB	37°C, 54 h	-	60°C, pH 4.5	24.5 U/mL	(Song *et al.*, 2016)
P. pastoris	Extracellular	pPIC9K with P_{AOX1} and α-factor PS	BMGY and BMMY	30°C, 48 h	-	60°C, pH 4.5	350.8 U/mL of supernatant fluid	(Xu *et al.*, 2006)
***Thermotoga maritima* MSB8 (90 kDa, 843aa)**								
B. subtilis	Extracellular	pWB980 : P43 promoter and SacB SP	LB	37°C, 12 h	Salting out and dialysis	90°C, pH 6.0, 5 mmol/L of Li^{+}, Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}. Inhibitors: Cu^{2+}, Fe^{2+}, EDTA, Zn^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+} and Al^{3+}.	89.1 U/mL of semi purified enzyme	(Su *et al.*, 2010)

Desulfurococcus mucosus* (*66 kDa, 668 aa)								
B. subtilis	Extracellular	pJA803 with maltogenic α-amylase promoter	BPX	37°C	Series of affinity chromatography steps and dialysis	85°C, pH 5.0	0.34 U/mg of protein in the supernatant fluid 26 U/mg of proteins in purified fraction	(Duffner *et al.*, 2000)
Bacillus stearothermophilus* (*62 kDa)								
B. subtilis	Extracellular	pTB522	LS	37°C	Salting out, dialysis, series of gel filtration steps and affinity chromatography	60 to 65°C, pH 6.0	0.09 U/mL of supernatant fluid 1.01 U/mg of protein in purified fraction	(Kuriki *et al.*, 1988)
***Geobacillus thermoleovorans* NP33 (C-terminal truncated amylopullulanase gene, 150 KDa, 1355aa)**								
P. pastoris	Extracellular	pGAPZalphaA with P_{GAP} and α-factor PS	BMGY	30°C, 250 rpm, 96 h	Ultrafiltration, affinity, chromatography	80°C, pH 8.0, Ca^{2+}	33.5 U/mL with specific activity of 286 U/mg	(Nisha and Satyanarayana, 2017)
P. pastoris	Extracellular	pPICZalphaA with P_{AOX1} and α-factor PS	BMGY and BMMY				28.6 U/mL with specific activity of 258 U/mg	
E. coli	Intracellular	pET28a(+) with T7 and the original SP, fusion Histidine tag	LB, 1.0 mM IPTG	25°C, 16 h	Cell lysis and affinity chromatography	60°C, pH 7.0, Ca^{2+}	specific activity of 1260 U/mg	(Nisha and Satyanarayana, 2013b)

Footnote: */ LB (Luria–Bertani Culture media): 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl; */ TB: 24 g/L tryptone, 48.00 g/L yeast extract, 2.31 g/L KH_2PO_4, 9.85 g/L K_2HPO_4, 25 g/L glycerol, and pH 7.0 (25 % ammonia water and 17 % H3PO4); */ BPX: suspension of 100 g of potato flour, 50 g of barley flour, 0.1 g of BAN 5000 SKB, 10 g of sodium caseinate, 20 g of soy bean extract, 9 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.1 g of pluronic acid per liter (final volume). The starting pH of BPX medium was 7.0; */LS: 2 g of tryptone, 2 g of yeast extract, 2 g of NaCl, and 1% of soluble starch; */ BMGY: 1% yeast extract, 2% peptone, 1% glycerol, 1.34% yeast nitrogen base, 0.00004% biotin, pH 6.0, and 10% 1 mol/L potassium phosphate buffer; */BMMY: BMGY with 1% methanol instead of glycerol, with supplementation of 0.5% methanol per each 24 h.

2.2.2. Advances in the production of thermoduric pullulanases by wild-type microorganisms

Production of thermoduric enzymes directly by wild-type thermophilic (50-80°C) and hyper-thermophilic (80-110°C) microorganisms presents several advantages, at least in theory. Important advantages are: low bacterial and viral contamination risks compared to mesophilic microorganisms, and high genetic stability compared to production employing recombinant microbial hosts (Fujiwara, 2002). Nevertheless, the high energy consumption needed to maintain a high temperature and to reduce evaporation during fermentation is a key factor preventing or limiting the use of thermophilic microorganisms at industrial scales. Moreover, fermentation processes based on thermophilic and extreme thermophilic wild-type microorganisms are characterized by low cell densities, thus, by low productivities and, consequently, by low enzyme titers (Gupta *et al.*, 2014).

Some studies have reported on potential advances aimed at overcoming the above limitations, either by designing bioreactors resistant to high temperatures, by adopting a cyclic fed-batch mode of operation or by using bioreactors with membranes (microfiltration and dialysis), in the hope of increasing cell density, *via* continuous supply of fresh culture medium and/or *via* elimination of toxic or inhibitory metabolites (Krahe *et al.*, 1996). In the case of fermentations involving bacteria of the *Sulfolobus* genus, the cell density increased to 132 g/L (DCW: dry cell weight) when one of those fermentation strategies was used (Schiraldi *et al.*, 1999). However, in some cases, the increase in cell density was not concomitantly paralleled by an increase in productivity with respect to the targeted enzymes (Antranikian *et al.*, 1987).

Many thermophilic and hyper-thermophilic microorganisms are known to produce thermoduric pullulanases. Table 2.1 presents some of these producers. Some of these thermoduric pullulanases are attached to the microbial cell surface (Domań-Pytka and Bardowski, 2004), which is a significant drawback for economical enzyme production. This characteristic may be explained by the fact that these enzymes have multiple transmembrane domains, leading to "sequestration" of the particular enzyme in/on the membrane (Choi and Cha, 2015). It was observed that the N-terminal cysteine residues are modified by covalent attachment to fatty acids, a possible explanation for anchoring of the enzyme to the cell membrane (Janse and Pretorius, 1993).

Some studies have used surfactants, such as t-octylphenoxypolyethoxy ethanol (Triton X-100), 3-[(3-cholamidopropyl), dimethylammonia]-l-propane sulphonate (CHAPS),

polyoxyethylenesorbitan mono-oleate (Tween 80), or sodium taurocholate, to "solubilize" enzymes, namely the *Sulfolobus acidocaldarius* amylopullulanase (optimal temperature and pH: 95°C and 3.0, respectively) the *Thermus aquaticus* pullulanase (optimal temperature and pH: 85°C and 6.5, respectively) (Plant *et al.*, 1986), the *Rhodothermus marinus* pullulanase (optimal temperature and pH: 80°C and 6.5, respectively) (Gomes *et al.*, 2003), and the *Clostridium thermosulfurogenes* pullulanase (optimal temperature and pH: 80°C and 6.0, respectively) (Reddy *et al.*, 1999b). Yield increases of 114, 146, 47, and 28% of the *Clostridium thermosulfurogenes* pullulanase, a high thermoactive and thermostable cell-bound enzyme, were obtained when Triton X-100, CHAPS, Tween 80, and sodium taurocholate were, respectively, added to the culture medium (Reddy *et al.*, 1999b). The same authors indicated that surfactants also enhanced stability of the pullulanase. However, surfactant concentration and the time of addition affect greatly growth of the microorganism, thus, impact on enzyme production. These two factors should be considered when a surfactant will be used to stimulate production of an enzyme and/or recovery of the enzyme following its "extraction" or "solubilisation". A much better understanding of the mechanism of action of these surfactants is required in order to optimize their use at large scales.

In addition, strategies such as the use of starch-limiting continuous cultivation and/or optimization of the pH or of the dilution rate, may be valuable approaches for releasing a cell-bound pullulanase to the culture fluid. It was reported that 85% of the *Clostridium thermosulfurogenes* pullulanase was excreted to the culture fluid simply by adopting one of these approaches, as enzyme titers more than doubled (to 4.2 U/ml) after increasing the pH from 5.5 to 6.7 and after decreasing the dilution rate to 0.03/h (Antranikian *et al.*, 1987).

Pullulanase production has been studied both using submerged fermentation (SmF) and solid-state fermentation (SSF). In the last decade, SSF has gained attention due to several biotechnological advantages (*e.g.* high fermentation capacity, increased enzyme production as compared to SmF, lower catabolic repression and cost-effectiveness), Other advanced technologies have been explored such as consolidated bioprocessing (CBP) (Behera and Ray, 2016). One example of CBP is combining starch hydrolysis and fermentation, whereby either ethanol from starch or concentrated sugar syrups from starch could be produced in "one pot". Such "one pot" processes may be performed using either a single microorganism or a co-culture of two or more microorganisms, in one step, and witshout the need for externally supplied enzymes (Kim *et al.*, 2010). Up to date, as far as one knows, a CBP

approach has not been used for the purpose of producing starch-converting enzymes with thermophilic or hyper-thermophilic microorganisms.

Solid-state fermentation (SSF) has been explored to optimize the production of a *Clostridium thermosulfurogenes* pullulanase by using a response surface methodology, which is an effective and economical statistical technique (Reddy *et al.*, 1999a). These authors were able to produce the pullulanase at a level of 4829 U/kg just by combining an optimized culture medium (16.5% potato starch, 2.5% corn steep liquor, 14% pearl millet, and 0.015% ferrous sulfate) with optimal leaching conditions (contact time 30 min) (Reddy *et al.*, 2000). However, no investigation was done to study the feasibility of SSF for producing the enzyme at large scale. Also, to the best of our knowledge, there has not been a comparison between SmF and SSF regarding productivity.

Various substrates may be used to induce the production of a pullulanase, depending on the microorganism. The production cost of these enzymes could be minimized by using inexpensive carbon and nitrogen sources. Maltose was reported to be a better inducer than starch for pullulanase production by *Rhodothermus marinus* (Gomes *et al.*, 2003) and by a *Clostridium* sp. (Ramesh *et al.*, 2001). In contrast, starch was found to be better than maltose and, surprisingly, maltose could even repress production of the enzyme (Ramesh *et al.*, 2001). In the case of *Clostridium thermosulfurogenes*, potato flour (10 g/l) was the better inducer, followed by soluble starch and rice flour (Ramesh *et al.*, 2001).

Yeast extract and peptone are usually used as nitrogen sources (Hii *et al.*, 2012a; Swamy and Seenayya, 1996). Many studies have evaluated the effect of other nitrogen sources (organic and inorganic) such as cheese whey and cassava whey on the production of alpha-amylases. Different sources of carbon and nitrogen from local agricultural sources were tested for the production of the *Thermoactinomyces thalpophilus* pullulanase, a stable and active enzyme at 55°C and pH 7.0 (Odibo and Obi, 1990). These authors could produce pullulanase with a yield of 198 U/ml after 36 h by using a shake flask fermentation process with a culture medium composed of yam starch (2%), soybean meal (5%), K_2HPO_4 (0.1%), NaCl (0.1%), $MgSO_4$.7H20 (0.1%), $SrSO_4$ (0.092%), pH adjusted to 5.0. However, no scale up of the fermentation process was reported.

High temperature is a limiting factor in the production of thermoduric pullulanases by wild-type, aerobic thermophilic microorganisms, mainly when temperature is above 65°C. Such processes consume significantly more energy and are accompanied by a large decrease in

oxygen solubility; as a consequence, more energy must go for oxygen supplementation. Increasing oxygen (air) flowrate for supplementation leads to increased evaporation and to unpredictable modification of the culture medium. Ideally, the bioreactor should be designed in such a way that the evaporation rate would be lower, leading possibly to increased bioreactor-related costs. This might be a reason why only a few studies have been published on the optimization of the production of pullulanases by wide-type thermophilic microorganisms, and none of them reported on the feasibility of production at large scales.

2.2.3. Advances in production by microbial recombinant hosts

High productivity of extracellular protein production/accumulation will often require optimization at the molecular level as well as optimization of the protein production process. The choice of the appropriate expression system (recombinant host and expression vector) is a very crucial step in this process (Lambertz *et al.*, 2014). The most common microbial hosts used are bacteria (*e.g. E. coli, Bacillus subtilis*), and yeasts (*e.g. Pichia pastoris*, *Saccharomyces cerevisiae*), due to their many advantages, mainly genetic and large-scale background knowledge availability, non-pathogenicity, relative simplicity, and low cost of production (Wang *et al.*, 2014; Zhang *et al.*, 2007). These microorganisms differ significantly in their cell wall structure, their subcellular compartments for protein targeting and their protein secretion possibilities/capabilities (Amon *et al.*, 2004). The purpose of recombinant production is to obtain high and rapid expression of the gene(s) of interest together with high titers of the target protein. Many factors are known to prevent or complicate success of the overall fermentation process.

First of all, biosynthesis of the protein of interest is dependent on the codon-reading machinery in the recombinant host. In general, pullulanase genes contain rather rare codons leading possibly to translational errors, and this is why it is very important to optimize the gene sequence (*i.e.* codon optimization) according to the recombinant host envisaged for gene expression. In many cases, the refolding process of the newly synthesized protein is incorrect and/or some chaperonins needed for successful secretion will be absent, leading to protein aggregation and, subsequently, to accumulation of inactive inclusion bodies in the cytoplasm or in the periplasmic space (Su *et al.*, 2010). Many strategies have been developed and adopted to resolve this solubility problem: 1/ use of efficient expression systems, 2/ co-production of chaperonins, fusion partners, or foldase(s), 3/ optimization of the operational conditions, mainly during the induction phase, and, 4/ genetic engineering. Sometimes, the

use of a His-tag increases the solubility of the protein and it greatly facilitate its recovery by chromatographic techniques (Han *et al.*, 2013; Yang, 2013).

2.2.3.1. Vectors for soluble expression

It is very important to notice that there are only a few regulated promoters (inducible or constitutive) available for Gram-positive species, such as *Bacillus subtilis*, compared to those available and used with Gram-negative species such as *E. coli*.

*2.2.3.1.1. **E. coli*** expression systems

E. coli is usually the first choice for prokaryotic protein expression, due to its many advantages. However, two outstanding problems exist with pullulanase expression by this bacterium (1) low level expression of a soluble pullulanase and (2) poor extracellular secretion efficiency (A. Chen *et al.*, 2016). These outstanding problems can be explained by the features of the *E. coli* outer membrane which is thick, thus, protein secretion occurs *via* a specific protein transporter or *via* membrane permeabilization (Yan and Wu, 2013). In recent years, many studies have proposed strategies to improve soluble expression and secretion efficiency with *E. coli*. The best known expression promoters used with *E. coli* are: the lac/tac/trc system, the P_L system, the T7 system and the heat-shock (Hsh) system (Wu *et al.*, 2014). Zouari Ayadi *et al.,* (2008) reported that PelB, OmpA, PhoA, endoxylanase, and StII signal peptides (SP) can be used for efficient secretory production of recombinant proteins by *E. coli* but their secretory efficiency was proven only for proteins with a molecular weight under 50 kDa. It would be interesting to investigate the effect of these signal peptides on pullulanase secretion. Therefore, the high molecular weight and complicated structure of pullulanases may hinder the use of these signal peptides and even prevent soluble pullulanase production and its secretion.

Chen *et al.,* (2014) have shown the capacity of the T7 promoter to express an extracellular pullulanase gene from *B. acidopullulyticus* by *E. coli* (4.07 U/ml of supernatant fluid), but only when it was combined with the *lac* operator and the *lac* repressor. When the T7 promoter was used alone as a regulatory system in the expression cassette, the production of pullulanase from *Anaerobranca gottschalkii* by *E. coli* was intracellular, which required cell lysis and chromatography work for enzyme recovery (Bertoldo *et al.*, 2004). On the other hand, in the case of expression of a pullulanase from *Thermococcus kodokarensis* by *E. coli*, when a His-tag was fused to the gene, production was extracellular (Han *et al.*, 2013). Actually, the combination T7lac promoter-operator and lacI repressor led to an efficient decrease in basal expression and, thus, improved recombinant protein solubility (Chen *et al.*,

2014). The challenge of complete elimination of inclusion bodies still exists and more advances are needed to solve this problem, when it is seen as a problem.

2.2.3.1.2. B. subtilis expression system

Two important advantages of this Gram-positive bacterium explain why it is regularly a first choice for extracellular protein production. The first one is the lack of an outer membrane, leading to more efficient secretion (Yan and Wu, 2013). However, secreted proteins may also be displayed on the cell surface *via* anchoring proteins such as Blc or PgsA (Yan and Wu, 2013). The second advantage is the absence of a significant *bias* in codon usage (no redundancy), which allows for efficient transcription and translation of the target protein (Su *et al.*, 2010). Many expression cassettes with various regulatory elements (promoters and signal peptides) have been developed and optimized for enhancing the expression of foreign genes. To produce the extracellular pullulanase from *B. naganoensis* using *B. subtilis*, Wang *et al.,* (2014) have tested twelve expression cassettes combining three promoters (P43 (P_{P43}), P_{apr} from *B. alcalophilus*, and P_{amy} from *B. amyloliquefaciens*) and four signal peptides (levansucrase SP_{sacB}, SP_{aprl}, SP_{aprs} from *B. alcalophilus*, and SP_{amy} from *B. amyloliquefaciens)*. The best expression cassette, yielding maximal activity in the soluble fraction (0.24 U/mg of total protein), contained P_{apr} and SP_{sacB}.

The major limiting factors for the use of *B. subtilis* as expression system are: 1/ structural instability of the recombinant plasmid due to its replication mode and 2/ instability of the protein produced due to its degradation by proteases during the fermentation process (Nguyen *et al.*, 2005). To solve the instability issue, some publications have reported on the construction and use of expression vectors based on the *E. coli-B. subtilis* shuttle vector pNDH37, which contains the *groESL* operon (the essential heat shock proteins (hsp) GroES and GroEL) of *B. subtilis,* fused to the *lac* operator, and the signal sequence of the *amyQ* gene. This vector exhibited structural stability and protein excretion capacity (Nguyen *et al.*, 2005). So far, a series of protease-deficient strains of this same host (*e.g.* WB600) have been constructed to solve the problem of degradation of the targeted protein by proteases. The thermoduric and acidoduric pullulanase from *B. naganoensis* was extracellularly produced by *B. subtilis* (Song *et al.*, 2016). Productivity increased from 8.7 U/ml to 24.5 U/ml simply by switching strains (from the WB800 strain to the WB600 strain, a protease-deficient one) while using the same expression cassette pMA09011, which contained the constitute promoter P_{43} and the lipase A signal sequence (Song *et al.*, 2016). This expression cassette may be used either in *E. coli* or in *B. subtilis*.

2.2.3.1.3. P. pastoris expression system

This methylotrophic yeast is presently used as recombinant host in several industrial applications. Li *et al.,* (2013) have indicated that *P. pastoris* was able to express genes that could not be efficiently expressed by bacteria, by the yeast *Saccharomyces cerevisiae* or using the *Baculovirus* system. The yeast *P. pastoris* has two great advantages: (1) an efficient secretory system and (2) a capacity to express genes at high levels (M. Ahmad *et al.*, 2014; Beaulieu *et al.*, 2005). It was observed that the amylopullulanase produced by *P. pastoris* displayed higher substrate specificity and stability than that produced by *E. coli* due mainly to post-translational modifications such N- and O- glycosylation (Nisha and Satyanarayana, 2017).

Three strong promoters are used for recombinant gene expression by *P. pastoris*: the alcohol oxidase 1 gene promoter (P_{AOX1}) (M. Ahmad *et al.*, 2014), the glyceraldehyde-3-phosphate dehydrogenase gene promoter (P_{GAP}) (Potvin *et al.*, 2010), and the formaldehyde dehydrogenase gene promoter (P_{FLD}) (Resina *et al.*, 2009). Table 2.2 compares the characteristics of these three promoters (M. Ahmad *et al.*, 2014; Nakano *et al.*, 2006; Potvin *et al.*, 2010; Resina *et al.*, 2009; Zhang *et al.*, 2009). Nisha and Satyanarayana (2017) have compared inducible *versus* constitutive expression of the amylopullunanase gene of *Geobacillus thermoleovorans,* combined with secretion, by *Pichia pastoris* (Table 2.1). They observed that constitutive GAP-based expression led to higher pullulanase production in comparison to inducible AOX1-based expression. A similar observation has been made for plectasin, an antibiotic substitute (X. Chen *et al.*, 2016). Nevertheless, the P_{AOX1} promoter has been the most widely used until now (Nisha and Satyanarayana, 2017). However, the use of this promoter may imply the use of relatively large quantities of methanol, during large-scale fermentation, which constitutes a potential fire and safety hazard (Macauley-Patrick *et al.*, 2005). Also, since methanol is still mainly derived from petrochemical sources, the use of methanol might be unsuitable in the production of food products and additives (M. Ahmad *et al.*, 2014; Nakano *et al.*, 2006).

Yeast-based expression systems are especially of interest as CBP organisms. However, to the best of our knowledge, only three publications are available regarding the use of *P. pastoris* to produce various pullulanases: that of *Bacillus naganoensis*, the isopullulanase from *Aspergillus* under the control of P_{AOX1}, and the amylopullunanase of *Geobacillus thermoleovorans* under the control of P_{AOX1} and P_{GAP}. Xu *et al.,* (2006) reported that the *Bacillus naganoensis* pululanase gene, when expressed in *P. pastoris* SMD1168 (Mut+, His-

, pep4-), using the pPIC9K vector, led to successeful extracellular production of the enzyme , at a level estimated at 350 U/mL, which was 1358% more than the production level obtained with *B. subtilis*.

The choice of the signal peptide (SP), located at the N-terminal portion of the protein, is a crucial step, allowing for the protein to cross through the endoplasmic reticulum (ER), where the SP is removed, thus, leading to efficient secretion (Delic *et al.*, 2013). The alpha-factor prepro-peptide of *S. cerevisiae* is widely used as secretion signal (Macauley-Patrick *et al.*, 2005). Effectively, the alpha-factor prepro-peptide of *S. cerevisiae* present in the pPIC9 vector improved more than 15-fold the extracellular production of the isopullulanse of *Aspergillus* by *P. pastoris* GS115, compared to the original signal peptide of the same enzyme (Akeboshi *et al.*, 2003).

The SP is composed of three domains: (1) The N-domain, positively charged, which reacts with the chaperonins associated with the translocation mecanism (2) the hydrophobic H-domain, which allows for the SP to be inserted into the ER membrane by forming a hairspin structure, and (3) the C-domain which allows the SP to be removed when the protein is in the ER lumen (Delic *et al.*, 2013). Some researchers have reported that the N-terminal domain of the enzyme and the number of its amino acids are responsible for its secretion (Brocca *et al.*, 1998). According to Venturini *et al.,* (1997), the N-FPTALVPR-C octapeptidic sequence, present in the N-terminal domain of the glucoamylase II of *Saccharomyces diastaticus* and in the β-galactosidase of *E. coli*, is responsable for the secretion of these proteins. Pechan *et al.,* (2004) observed that changing of the N-terminal domain of the pullulanase could prevent its secretion, even if the SP used is well known for its secretion efficacy.

Considering expression stability, loss of the recombinant plasmid would be an important issue with recombinant hosts, and supplementation of the selected antibiotic during fermentation would be often helpful to maintain the positive transformant (Song *et al.*, 2016). However, the use of antibiotics at large scale is not recommended and is often unfeasible, due mainly to potential environmental issues, increase in processing costs, and development of resistance, including resistance by the recombinant host itself. There is a need to develop new marker genes, where the marker will be not harmful to the environment.

Finally, it is important to remember that, whatever the genetic engineering strategy used, the success in extracellular enzyme accumulation may often be directly linked to the success in

transferring the desired protein from the cytoplasmic space to the extracellular medium. Structural features of the protein and individual interactions among these features will often influence the protein's function and they will play a key role in the efficient extracellular production of a soluble and active thermoduric pullulanase.

2.2.3.2. Effect of process conditions on the soluble expression of pullulanase

Many parameters influence the solubility of a newly synthesized pullulanase, mainly cell growth, type and feeding strategy of the inducer, duration of induction, pH and temperature during induction, and concentration of the added osmolytes (Duan *et al.*, 2013b). The concentration of a given desired protein accumulating in either the extracellular fraction or in the intracellular fraction should be roughly proportional to the concentration of cells in the culture, and this is often the case. It is desirable to eliminate complex ingredients (*e.g.* yeast extract, peptone and tryptone) from the culture medium in order to gain full control on medium composition, and to develop a robust and reproducible standardized production protocol, one that will be easily validated. Some authors have even reported that intracellular or extracellular protein production/accumulation might be more carbon source-dependent than promoter-dependent (Hohenblum *et al.*, 2004). To the best of our knowledge, attempts to optimize recombinant pullulanase production have been made using solely *E. coli* as expression host. A decrease in culture temperature facilitated proper folding and, thereby, improved pullulanase production. It was shown that decreasing the temperature during induction from 20°C to 16°C enhanced *B. acidopullulyticus*-derived extracellular pullulanase production by *E. coli* (T7lac-lacI), increasing from 4.07 U/mL to 11.30 U/mL. Furthermore, integration of a feeding strategy in order to increase cell density, in combination with a step involving a decrease in temperature during the induction phase, improved significantly extracellular production, from 11.30 U/mL (shake flask fermentation) to 1156.32 U/mL (bioreactor fermentation) after incubation for 28 h (Chen *et al.*, 2014). In general, the feeding strategy proposed by many studies is performed in three phases: batch phase, fed-batch phase, during which the cell density increases sharply, and induction phase.

The choice of the inducer is also important. In the case of the T7 promoter, both lactose and isopropyl β-D-1-thiogalactopyranoside (IPTG) are used as inducer. Nevertheless, lactose improved pullulanase yield more effectively than IPTG, and it also promoted growth of *E. coli* (Zou *et al.*, 2014). Furthermore, a lower concentration of the inducer had a positive effect on the solubility and proper folding of proteins. The optimization of extracellular

production of the pullulanase from *B. deramificans* by *E. coli,* using the expression shuttle pET20b(+) with the pelB signal peptide, was carried on by Duan *et al.,* (2013b) who showed that, by decreasing growth temperature from 37°C to 30°C, decreasing the induction temperature from 30°C to 25°C, and decreasing the IPTG concentration down to 0.05 mM, pullulanase activity increased from 2.1 U/ mL to 13 U/mL.

E. coli contains a network of chaperonins composed of proteins involved in translocation, refolding and excretion of other proteins, which requires activation by external and internal compounds. It was found that an increase in the concentration of some natural osmolytes can specifically activate ClpB (a protein belonging to the chaperonin disaggregation network) under combined salt- and heat-stresses, resulting in an decrease of inclusion body formation (Diamant *et al.*, 2003). Duan *et al.,* (2013b) have used this feature to evaluate the effect of some natural osmolytes (betaine, proline and K-glutamate) on the solubility of pullulanase. When 20 mM of betaine was added two hours prior the induction, enzyme accumulation in the periplasmic space was improved (45.3 U/mL) but not that in the supernatant fluid.

However, it is rather difficult to extract enzymes from the periplasmic space, especially at larger scales, and the cost is usually high. Thus, simple and low-cost approaches have been proposed in the literature. The first one is the use of glycine as a medium supplement in order to enhance permeability of the cell's outer membrane. Zou *et al.,* (2014) have used this approach to produce the pullulanase from *B. deramificans* by *E. coli*. The OmpA signal peptide was tested instead of the pelB signal peptide, and glycine at 1% was added to the culture media. Extracellular pullulanase activity was 8.3 times higher than that of the control. When glycine is supplemented during the exponential phase, it can replace L-alanine and D-alanine in the cell wall's peptiglycan leading to a more loosely cross-linked peptidoglycan, which means cells become more permeable. However, it is important to notice that a glycine concentration higher than 0.5% inhibited growth of *E. coli*. Some other authors have tested non-ionic surfactants, such as Triton X-100, due to their dual capacity to solubilize pullulanase aggregates in the periplasmic space and to improve cell permeability (Duan *et al.,* 2015). Duan *et al.,* (2015) improved by 46-fold the secretion ratio by using 0.5% Triton X-100 after 24 h of induction.

Table 2.2: Characteristics of using P_{AOX1}, P_{FLD} and P_{GAP} in the production of recombinant proteins by *P. pastoris*.

Promoter	**P_{AOX}**	**P_{FLD}**	**P_{GAP}**
Type of expression	Inducible	Inducible	Constitutive
Preferred carbon source	Glucose and glycerol	Glucose and glycerol	Glucose
Inducer	Methanol	Methylamine	None
Safety	(-)	(+)	(+)
Cellular stress	(+)	(+)	(-)
Repressor of the promoter	Glycerol and ethanol	-	-

Footnote: (-) less, (+) more.

This table compares P_{AOX1}, P_{FLD} and P_{GAP} in the production of recombinant proteins by *P. pastoris*. The change of substrate to induce P_{AOX1}, and P_{FLD} to produce the enzyme, yields more stress for the cells compared to the production of enzyme by using constitutive expression. Also, the use of methanol in the case of **P_{AOX}** presents some safety issues such as explosion and health problems for workers.

2.2.4. Advances in protein engineering to improve the thermostability and the solubility of pullulanases

Thermostability of pullulanases has been considered as a requirement for their industrial use in order to maintain high catalytic activity under high temperatures. Many studies have focused on the molecular structure (3D) of thermozymes and to compare it to that of their mesophilic equivalents. It has been reported that the similarity between their protein sequences is 40 to 85%, and that they have the same catalytic mechanisms (Vieille and Zeikus, 2001). It has been reported also that hyper-thermophilic proteins exhibit significantly reduced hydrophobic accessible surface areas compared with mesophilic proteins (Li *et al.*, 2015). Moreover, their structures showed one or several proline residues located in the surface's loop region (Farhat-Khemakhem *et al.*, 2013). Indeed, proline residues reduce the thermal flexibility of the loop, thus, generating high thermostability (Farhat-Khemakhem *et al.*, 2013).

Protein engineering can be an efficient approach to increase the thermostability of an enzyme and its catalytic activity, mainly by combining three major factors: 1/increasing the external surface charge by increasing hydrogen bonds and ionic interactions; 2/increasing the rigidity of the refolding zones of the protein by adding multiple pair-ion networks; and 3/increasing the hydrophobicity of the protein envelope (external 3D strucutre) (Elleuche *et al.*, 2014; Kumar *et al.*, 2011). Generally, protein engineering may be classified into two categories: rational design and irrational design (called also directed evolution) (Duan *et al.*, 2013a).

The first step in the protein engineering process, despite whichever approach is adopted, is to probe the protein's 3D structure, and its complex modular architecture, in addition to solving its substrates/protein complex structure. Investigations on these various aspects help in the development of more powerful biocatalysts for demanding environmental-friendly processes (Turkenburg *et al.*, 2009).

2.2.4.1. Molecular structure of pullulanases

In general, GH13 pullulanases show multi-domain architectures: */ a **pullulan-degrading enzyme N-terminus domain** containing some motifs with unknown function, and several carbohydrate-binding modules (CBMs), except for some pullulanases such as the *Klebsiella pneumoniae* UNF5023 pullulanase, whose N-terminal domain does contain CBMs, and the *Anoxybacillus sp.* LM18-11 pullulanase whose N-terminal contains only one CBM; */ **the α-amylase catalytic $(\alpha/\beta)_8$ barrel core (A domain)** consisting of four highly conserved regions that are implicated in catalytic activity, substrate binding and metal ion binding

(Albertson *et al.*, 1997). The A domain contains Asp-206, His-210, and His-296, which are important for substrate binding, and the catalytic residues Glu and Asp (De Souza and Oliveira Magalhães, 2010). The A domain of the GH13 family is closely linked to protein stability (Deng Z. *et al.*, 2014) ; and */ **the C-terminal region** containing one α-amylase C-terminal domain (AamyC), which helps the enzyme in binding specifically with the insoluble substrate, plus various fibronectin type III (FnIII) domains, and CBMs (Lin *et al.*, 2012).

CBMs are currently divided into 39 families based on amino acid sequence similarity, and they have different ligand specificities for recognizing crystalline cellulose, non-crystalline cellulose, chitin, beta-1,3-glucans and beta-1,3-1,4-mixed linkage glucans, xylan, mannan, galactan and starch (Lin *et al.*, 2008). Fujiwara (2002) reported that all amino acid sequences of pullulanases type 1 have the conserved active center Tyr-Asn-Trp-Gly-Tyr-Asp-Pro, which is responsable for binding to substrate and for the catalytic reaction. This kind of enzyme possesses also several modules with unknown function, which may be modified during any protein engineering process (Turkenburg *et al.*, 2009).

Each pullulanase has affinity for a particular substrate (Saha and Zeikus, 1989). For instance, the *Aerobacter aerogenes* pullulanase presents an activity of 100% for pullulan, 164% for amylopectin β-limit dextrin, 172% for amylopectin α-limit-dextrin, and less than 1% for liver glycogen (Mercier *et al.*, 1972). Contrary to the *Thermococcus kodakarensis* pullulanase which presents an activity of 100% for pullulan, 76% for cyclodextrins, 60% for potato starch, 37% for amylopectin, 26% for glycogen, and 5% for β-cyclodextrins.

As far as one knows, no structural information about the site recognizing the α-D-1,6 glycosidic linkage within a pullulanase is available. Especially, the fact that the pullulanase is a specific enzyme for oligo- and polysaccharides with specific structures, as mentioned at the beginning of this review. Also, there are two questions unanswered yet: 1/ why the pullulanase type 1 cannot hydrolyze the α-D-1,4 glucosidic bond, even if its structure contains the alpha-amylase A domain? 2/ what are the structural features of alpha-amylases and glucoamylases that make them much more active (high catalytic efficiency) than pullulanases?

To complete this section, it is worthwhile to list some of the tools used to modulate the 3D structure of enzymes. The Protein Data server (PDB, http://www.rcsb.org/pdb/home/home.do) provides the 3D protein structures presently available, and the theoretical structure of any new protein could be obtained *via* homology

modeling using the SwissModel Protein-Modeling server (https://swissmodel.expasy.org). ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2) is usually employed for multiple sequence alignment. The Synchrotron Radiation Facility (X-ray crystallography) is often used to elucidate the crystal structure of proteins (Turkenburg *et al.*, 2009). Once the 3D structure is determined, some software such as PYmol ((http://www.pymol.org) may be utilized for more structural analyses.

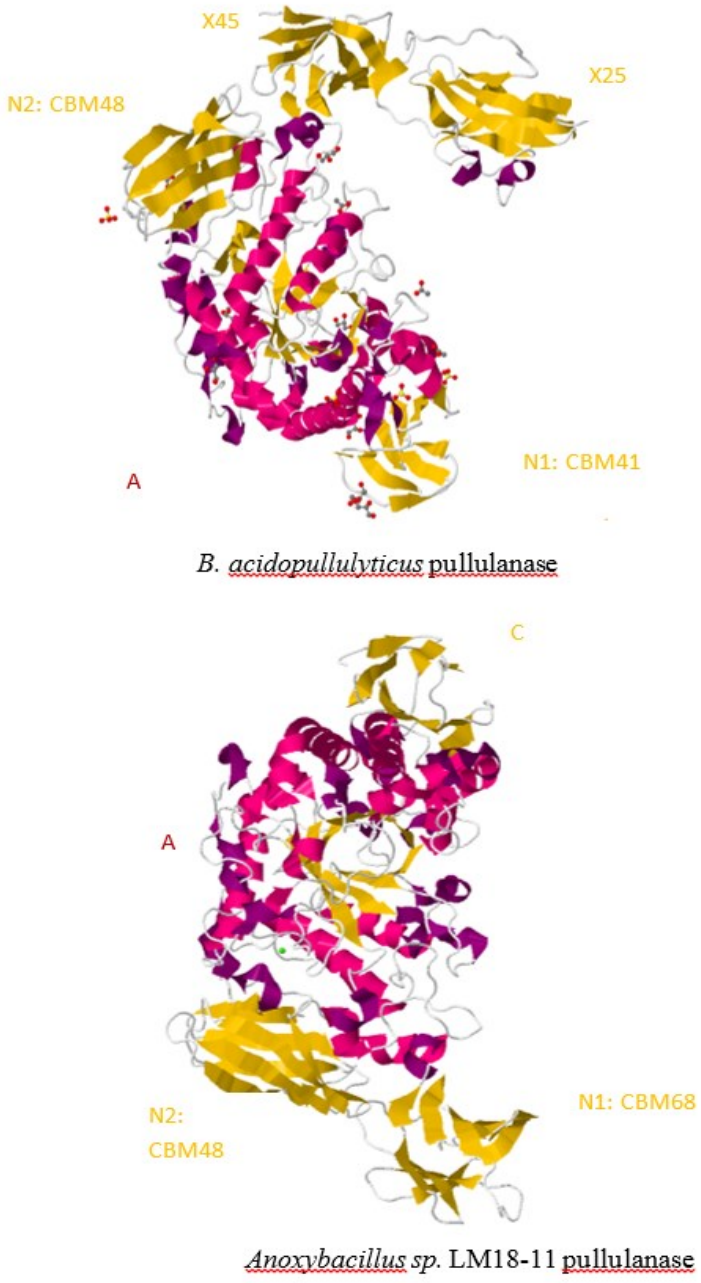

Figure 2.3: 3D structures of *B. acidopullulyticus* pullulanase (PDB code 2WAN) (Turkenburg *et al.,* 2009) and *Anoxybacillus sp*. LM18-11 pullulanase (PDB code 3WDH) (Xu *et al.*, 2013) and their different domains A (purple surface), N1, N2 and C (Yellow surface).

This figure compares two 3D structures of thermoduric pullulanases provided by RCSB Protein Data Bank (www.rcsb.org/pdb), and their domains. There domains are represented by color. They have the same domains A and N2, however their N1 domains are different. *B. acidopullulyticus* pullulanase N1 domain belongs to the CBM41 family, and *Anoxybacillus sp.* LM18-11 pullulanase N1 domain belongs to the CBM68 family, which could explain why the catalytic activity was eliminated for *Anoxybacillus sp.* pullulanase and yet remained for the *B. acidopullulyticus* pullulanase when N-domain were truncated.

2.2.4.2. Directed evolution

To improve secretion efficiency, in many studies, it was found that decreasing the molecular weight of the enzyme *via* domain truncations was a solution. An active pullulanase can probably be obtained from the precursor molecule after protein processing, such as proteolytic cleavages at the N- or C-terminal region of the protein. The enzymatic properties of such truncated proteins may be changed, however, due to the newly introduced molecular structure modifications.

The N-terminal section is not always essential for industrial applications and it has been shown that protein truncation did not affect the debranching function of the enzyme against low molecular weight dextrins (Teague *et al.*, 2001). Chen *et al.,* (2016) have used this approach to decrease the molecular weight of the pullulanase of *Bacillus acidopullulyticus* (101 kDa) without affecting catalytic activity. Truncation of the carbohydrate-binding domain CBM41, which resulted in an 89 kDa protein, increased soluble expression, hence, increased the production yield by 189%. Moreover, the protein variant exhibited better thermostability (82°C) than the wild-type enzyme (70°C). However, the same authors did not evaluate the effect of N-terminal truncation on the secretion of the engineered pullulanase. As it was mentioned earlier in this this paper, N-terminal domain modification may limit secretion of the enzyme. Wu *et al.,* (2014) reported that the *Thermus thermophilus* HB27 pullulanase lost its catalytic activity when 60 amino acid residues form the N-terminal domain were removed. Furthermore, when Xu *et al.,* (2014) eliminated CBM68, located at the N-terminal region of the *Anoxybacillus sp.* LM18-11 pullulanase, the resulting mutant was characterized by less thermostability and less catalytic activity in comparison to the wild-type pullulanase. These authors confirmed the importance of the N-domain for thermostability and catalytic activity (Xu *et al.*, 2014). These contradictory observations could be explained by the fact that pullulanases contain several CBMs belonging to different families, which implies different interactions between CBMs and other protein domains, mainly the A domain. Figure 2.3 compares the 3D structure of two pullulanases, one from *B. acidopullulyticus* and one from *Anoxybacillus sp.*

Moreover, the C-terminal motifs of CBM20 and of one FnIII domain (up to 100 amino acid residues) of the pullulanase from *Thermoanaerobacter pseudoethanolicus* have been truncated to evaluate and understand the roles of these modules in pullulanase activity; these domains were not found essential (Lin *et al.*, 2008). Truncation of the end region of the C-terminus (300 amino acid residues) of the *G. thermoleovorans* amylopullulanase revealed

that this region was also not essential for substrate binding and stability of the enzyme (Nisha and Satyanarayana, 2013b). However, a C-terminal region may contain a particular peptide recognized by the secretory machinery leading to efficient transport of the pullulanase outside the cell membrane (Lin *et al.*, 2012). Furthermore, removal of some domains exhibited no effect on catalytic activity, despite their role in facilitating folding of the protein into the correct conformation (Chen *et al.*, 2016).

The main drawbacks of a directed evolution approach are the requirement for large libraries and the need for many mutagenesis cycles in order to find improved mutants, and the usual lack of highly efficient methods for high throughput screening (Li *et al.*, 2015).

2.2.4.3. Rational design

This approach is considered as an efficient and reliable one to improve enzymatic properties. Currently, there is no universal method to rationally design enzymes, due to the great diversity of the factors that influence thermostability and catalytic activity (Li *et al.*, 2015). Some of the most prominent computational tools used to reach these objectives are SHEMA, ProSAR, and ROSETTA (Bommarius *et al.*, 2011). Two approaches have been explored to increase the thermostability of pullulanases: the structure-guided consensus approach and the data-driven rational design approach.

2.2.4.3.1. Structure-guided consensus approach

This approach combines information about quaternary structure with knowledge of a few enzyme sequences that share moderate identity in order to reduce the number of target residues in a given protein as candidates for mutational work (Vázquez-Figueroa *et al.*, 2007). Its bottleneck's are the lack of structural data in many cases and the limitation of tools needed to predict the influence of amino acid alterations on the stability and catalytic activity of the enzyme.

Recently, Duan *et al.,* (2013a) have used this approach to improve the thermostability of the *Bacillus deramificans* pullulanase, by substituting Asp-437 and/or Asp-503 by His-437, Phe-503 or Tyr-503. The choice of these residues was based on the *B. acidopullulyticus* pullulanase structure. The authors observed that, in the wild-type enzyme, there was no interaction between the side chain of Asp-437 and the side chain of Asp503, and the space around the two residues was large enough to form an internal cavity which may have contained water, thus, affecting stability of the *B. deramificans* pullulanase. By substituting these residues with His-437 and Tyr-503, van der Waals interactions between the imidazole

group of histidine and the benzene ring of tyrosine were promoted, and it led also to the formation of new hydrogen bonds with neighboring polar and charged atoms. The enzyme mutant was not just more thermostable (half-life of 95 h at 60°C and pH 4.5 compared with a half-life of 22 h for the wild-type enzyme under the same conditions) but its substrate-binding affinity was also improved. However, its catalytic constant was lower than that of other mutants, Duan *et al.,* (2013a) therefore assumed that proper conformational flexibility was critical to catalytic activity.

Part of this hypothesis was confirmed by Li *et al.,* (2015) who combined the structure-guided consensus approach with analyses of the residue water exposed surface method (ACCs) to improve the thermostabilty of the *Anoxybacillus sp.* LM18-11 pullulanase. These authors deleted the highly flexible regions of the pullulanase. The first region was made of six residues (1-6) at the N-terminus, and its deletion caused a reduction in the optimal temperature of 5°C, likely because of the presence of CBM68 which is important for the binding between the substrate and the enzyme. The second region consisted of the residues 686-688 in the C-terminal domain, which increased the thermostability of the resulting mutant by 1°C. To increase hydrophobicity of the protein, Li *et al.,* (2015) chose to substitute amino acids with ACC values superior to 40, using DSSP (http://www.cmbi.ru.nl/xssp/), with those proposed by the consensus approach (substitution of Y447, Y175, L215 and R473 with A447, C175, P215 and E473, respectively). They obtained a mutant more thermostable than the wild-type pullulanase. It retained 66% of its initial activity after incubation at 60°C for 72 h, whereas that of the wild-type enzyme was only 35%.

2.2.4.3.2. Data-driven rational design approach

The aim of this approach is to predict optimal amino acid substitutions by using computational tools such as the SCHEMA and RONN softwares, which substitutions could theoretically improve thermostability of an enzyme (Heinzelman *et al.*, 2009). Usually, a small library of mutants (less than 100) is built (Bommarius *et al.*, 2011). Chen *et al.,* (2015) have explored this approach to improve thermostability of the *B. acidopullulyticus* pullulanase. The methodology used by these authors was generation of mutants by different methods: identification of flexible regions by determining the high B-factor (or the temperature factor) values of the enzymes, identification of surface residuals with solvent accessibility higher than 50%, use of a consensus approach to substitute amino acid residues with the most prevalent amino acids in thermoduric pullulanases, and, finally, introduction of proline residues in the region loops of the A domain. The most stabilized single-site

mutant was E518I, which was characterised by a K_{cat}/K_m value equal to 2038 ml/mg*s, and a specific activity of 1158 U/mg, while keeping 86% of its initial activity after incubation at 60°C for 30 min. In comparison, the wild-type enzyme gave the following values: K_{cat}/K_m of 1473 mL/mg*s and a specific activity of 1096 U/mg, while retaining 55% of the initial activity at 60◦C for 30min. The single site selections were combined to build additional mutants, with some of them characterised by high thermostability (*e.g.* mutant E518I-S662R-Q706P) compared to the wild-type enzyme. However, their calculated catalytic efficiency and specific activity were lower than those of the wild-type pullulanase.

In summary, the success of protein engineering for developing a thermoduric enzyme with high catalytic efficiency still depends on our understanding of the contributions of amino acid residues and their interactions and on their overall influence on protein dynamics. It may be concluded from these studies that, to increase the thermostability of a pullulanase, it is important to increase the rigidity and the hydrophobicity of the protein. However, these increases favour protein compactness but reduce catalytic efficiency, resulting from the modifications made to the binding and catalytic sites and/or to a lower accessibility for the substrate to these sites. This fact may explain why hyperthermostable pullulanases have limited activity at high starch concentration, which still limits their application in industry.

The perfect industrial biocatalyst should have high specific activity, high substrate specificity and be stable under industrial process conditions (Bommarius *et al.*, 2011). The challenge now is to develop computational and experimental tools capable to predict the effect of amino acid substitutions on thermostability, specificity, and catalytic efficiency, and capable also to identify with significant exactitude mutants that should be stable and highly active.

2.2.5. Concluding remarks

Thermoduric pullulanase production, to be economical and to generate a product that will be used in various industrial applications, must be efficient and must favour accumulation of the enzyme in the culture's extracellular fraction. The progress towards better pullulanase production reviewed in this document reveals that two major approaches may be employed to meet this important challenge. Firstly, many research teams have used a heterologous expression approach to overcome the difficulties in production of these enzymes by wide-type strains. The choice of the recombinant host and of the expression vector (plasmid structure, promoter, signal peptide, etc.) is a critical aspect that should be seriously

considered in any enzyme production enterprise. Secondly, the elucidation of the structure of these proteins and their interaction and affinity with substrates are very important for success in the development of an ideal pullulanase for industrial applications, one that will be capable of maintaining an equilibrium between structural rigidity and functional flexibility. It is important to take into account, when protein engineering is being considered as an optimization approach, the fact that thermoduric pullulanases are complex and large proteins (52 – 110 kDa), and that some of them are active in their dimeric form, which tend to reduce greatly their extracellular accumulation.

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Chapitre 3. Est-ce que *Thermus thermophilus* HB8 peut être un producteur industriel d'amylases, principalement de pullulanases ?

Chapitre 3. Est-ce que *Thermus thermophilus* HB8 peut être un producteur industriel d'amylases, principalement de pullulanases ?

Avant-propos

Titre de l'article

Optimisation de la production extracellulaire d'enzymes amylolytiques thermostables par *Thermus thermophilus* HB8, et caractérisation basique.

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L'article présenté dans ce chapitre concerne le deuxième objectif du projet, à savoir l'étude de la capacité de *Thermus thermophilus* HB8 d'accumuler de façon extracellulaire des enzymes amylolytiques, principalement la pullulluanase type 1. Par l'exploitation de techniques statistiques à multi-variables, une optimisation des conditions opératoires fut réalisée, pour maximiser l'activité amylolytique extracellulaire produite par la bactérie thermostable.

Résumé :

L'objectif principal de ce projet est l'étude de la capacité de *Thermus thermophilus* HB8 d'être un producteur efficace d'enzymes amylolytiques thermostables. Les tests de production initiaux ont indiqué clairement que seulement de très faibles niveaux d'activité amylolytique pouvaient être détectés, à partir d'extraits cellulaires en utilisant le détergent doux non-ionique Triton X-100. Une stratégie d'optimisation séquentielle, basée sur des techniques statistiques, a été utilisée pour augmenter fortement la production d'une activité amylolytique extracellulaire afin d'obtenir des niveaux de production attrayants. L'accent a été mis sur le niveau optimal de la concentration initiale en biomasse, la composition du milieu de culture et la température pour maximiser l'accumulation d'enzymes amylolytiques.

Des modèles empiriques ont ensuite été développés, décrivant les effets des paramètres expérimentaux et leurs interactions sur la production d'enzymes amylolytiques. Suite à de tels efforts, l'accumulation d'enzymes amylolytiques extracellulaires a été augmentée de plus de 70 fois, avec des niveaux de production de 76 U/mL. La préparation brute d'enzyme extracellulaire a été partiellement caractérisée. Les valeurs optimales de température et de pH furent de 80°C et 9.0, respectivement. 100% de l'activité enzymatique initiale a pu être maintenu après 24 h d'incubation à 80°C, ce qui prouve la thermostabilité très élevée de la préparation enzymatique.

Mots clés

Thermus thermophilus HB8, enzymes amylolytiques thermostables, plan factoriel, plan composite central, fermentation.

Entête du manuscrit 2

Title

Optimization of the production of an extracellular and thermostable amylolytic enzyme by *Thermus thermophilus* HB8 and basic characterization

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Abstract

The objective of this study was to determine the potential of *Thermus thermophilus* HB8 for accumulating a high level of extracellular, thermostable amylolytic enzymes. Initial production tests indicated clearly that only very low levels of amylolytic activity could be detected, solely from cells after extraction using the mild, non-ionic detergent Triton X-100. A sequential optimization strategy, based on statistical designs, was used to enhance greatly the production of extracellular amylolytic activity in order to achieve industrially attractive enzyme titers. Focus was placed on the optimal level of initial biomass concentration, culture medium composition and temperature for maximizing extracellular amylolytic enzyme accumulation. Empirical models were then developed describing the effects of the experimental parameters and their interactions on extracellular amylolytic enzyme production. Following such efforts, extracellular amylolytic enzyme accumulation was increased more than 70-fold, with enzyme titers in the 76 U/mL range. The crude extracellular enzyme was thereafter partially characterized. The optimal temperature and pH values were found to be 80°C and 9.0, respectively. 100% of the initial enzyme activity could be recovered after incubation for 24 h at 80°C, therefore, proving the very high thermostability of the enzyme preparation.

Keywords

Thermus thermophilus HB8, thermostable amylolytic enzyme, factorial design, central composite design, fermentation.

3.1. Introduction

A good number of industrial processes employing depolymerases (chitinases, amylases, pectinases, and xylanases, etc.) are performed at high temperatures for better yields and lesser risks of bacterial and/or viral contamination (Fujiwara 2002; Kumar *et al.,* 2011). Such a requirement generates the need to develop novel enzymes that will be highly active and stable under rather harsh industrial processing conditions, which has led the industrial and scientific communities to devote time and efforts on thermophilic microorganisms as sources of such processing enzymes (van den Burg, 2003).

Generally speaking, following identification of a promising thermostable enzyme, researchers will adopt a heterologous gene expression approach to maximize production of the enzyme of interest *via* cloning of the necessary gene into a mesophilic microorganism (bacterium, yeast, and fungus). The most common microbial hosts used are *Escherichia coli*, *Bacillus subtilis, Saccharomyces cerevisiae*, and *Pichia pastoris* as well as various fungal species. Culture media and high cell density protocols are available today for producing recombinant enzymes in very high titers although it is also recognized that success with gene expression is still gene-dependent (Schiraldi and De Rosa, 2002; Morozkina *et al.,* 2010). Such an approach explains why optimization and scale-up of fermentation processes based on thermophilic hosts for producing such enzymes have received little attention so far and significant successes in this area are rare. Production of thermoduric enzymes directly by wild-type thermophilic (50-80°C) and hyper-thermophilic (80-110°C) microorganisms presents several advantages. Important advantages are low bacterial and viral contamination risks compared to mesophilic microorganisms, and high genetic stability compared to production employing recombinant microbial hosts (Fujiwara, 2002). Despite the highly attractive thermostability of these enzymes, their use in industrial processes is still quite limited and this is due mostly to low production yields, significant process difficulties and limited activity at high substrate concentrations (Wu *et al.,* 2014).

Amylolytic enzymes (amylases), belonging to the glycoside hydrolase family (GH), make up about 25% of the world's enzyme market (De Souza and Oliveira Magalhães, 2010). These enzymes hydrolyze the alpha-D-1,4 glycosidic bond and/or the alpha-D-1,6 glyosidic bond in starch and in related polysaccharides (Maalej *et al.,* 2013). They are used in several industrial fields (*e.g.* food, pharmaceutical, textile, detergents, paper, etc.) (Farris, 2009). The amylases used in starch transformation processes to yield glucose-, fructose- and maltose-concentrated syrups are alpha-amylases, beta-amylases, glucoamylases and

pullulanases (Plant *et al.,* 1986). These enzymes should ideally be highly active and stable at high temperature, mainly because the solubility of starch starts at 100°C, and in acidic conditions (pH 4.5 to 5.5) (Gomes *et al.,* 2003; Miller and Blum 2010). In the textile area, amylases are used to preserve fabric softness, and they should be stable and active under detergent conditions including alkaline pH (Maalej *et al.,* 2013).

Thermus thermophilus HB8, a recognized thermophilic Gram-negative eubacterium with significant biotechnological potential, shows promise for producing several thermostable enzymes (Cava *et al.,* 2009). It can also produce a polyhydroxybutyrate (PHB) polymerase enzyme (Pantazaki *et al.,* 2011) as well as a PHB depolymerase (Papaneophytou *et al.,* 2009). Berger *et al.,* (1995) have confirmed the ability of the HB8 strain to produce extracellular esterases, lipases and galactosidases (Berger *et al.,* 1995).

Genomic sequencing of the HB8 strain has been completed and the results show several genes corresponding to putative polysaccharide hydrolases (Table 3.1) but, so far, no experimental evidence of their expression and consequent enzymatic activity has been reported (Swarup *et al.,* 2014). Despite the presence of genes corresponding to putative amylopullulanase and neopullulanase enzymes, which hydrolyze the alpha-D-1,4 glycosidic bond of oligo- and poly-saccharides and of pullulan, respectively, Tomiyasu *et al.,* (2001) have shown that the HB8 strain was able to produce, at low level (0.016 U/ml), a pullulanase type 1 enzyme with a 80 kDa molecular mass, hydrolyzing the alpha-D-1,6 glycosidic bond of pullulan. The low enzymatic activity reported was most probably due, in part, to low cell density cultivation. To the best of our knowledge, there is still no published scientific report on the optimization of the production and accumulation of an extracellular amylolytic enzyme using this HB8 strain.

The primary objective of this study was to convert *T. thermophilus* HB8 into an industrially attractive microbial factory for accumulating high titers of an extracellular amylolytic activity. In the hope of achieving this objective, we have performed statistical optimization of key process parameters in order to identify the co-optimal levels for temperature, initial yeast extract concentration, initial tryptone concentration, initial NaCl concentration and initial biomass concentration, in addition to gaining an insight of the interactions among these factors. Additional objectives were: (1) verification of the capacity of the HB8 strain to produce pullulanase enzymes and (2) basic characterization of the thermostable, extracellular amylolytic enzyme produced by the HB8 strain.

Table 3.1 Putative polysaccharide hydrolases identified after genomic sequencing of *T. thermophilus* HB8 (NCBI Reference Sequence: NC_006461.1).

Putative Enzyme	Gene Code	Theoretical Isoelectric Point, pI*	Theoretical Molecular Weight, MW*	Size (amino acids)	Enzyme Family
Amylopullulanase	TTHA0158	5.39	112.2	994	GH57
Neopullulanase	TTHA1563	6.48	53.8	475	GH13
Amylomaltase	TTHA1261	5.60	57.2	500	GH77
Maltodextrin glucosidase	TTHA1647	6.89	63.2	551	GH13
Oligo-1,6-glucosidase	TTHA0481	5.63	61.4	529	GH13

* pI and MW calculated by using http://web.expasy.org/compute_pi/.

3.2. Materials and methods

3.2.1. Strain and growth conditions

T. thermophilus HB8 (ATCC 27634), received in a lyophilized state, was grown in a 500 mL-Erlenmeyer flask containing 100 mL of DSMZ-74 medium. Medium DSMZ-74 was made up of the following ingredients: 4.0 g/L of yeast extract (Fisher Scientific, BP-1422), 8.0 g/L of tryptone (Fisher Scientific, BP-1421), 2.0 g/L of NaCl (Fisher Scientific, S271), and pH adjusted at 7.0 using 2 M NaOH. The culture was incubated in a rotary shaker at 70°C with stirring at 200 rpm for 24 h. Sterile glycerol (50% (w/v) solution) was added to reach a final concentration of roughly 16% and the resulting culture was distributed in sterilized tubes (1.5 mL/tube) and stored at (-78 ± 2)°C. This constituted our Working Cell Bank (WCB). All subsequent experiments were initiated from this WCB by preparing a pre-culture inoculated by adding 0.8 mL of WCB cells per 100 mL of culture medium.

3.2.2. Cultivation of *T. thermophilus* HB8 on corn starch

The inoculum was prepared in DSMZ-74 medium and the culture was incubated at 70°C and at 200 rpm for 24 h. The medium used for testing for extracellular amylase production was the DSMZ-74 medium supplemented with 1% (w/v) corn starch (Sigma-Aldrich, S4126-2). Inoculum size was 5% (v/v). These batch fermentations were performed in 500-mL baffled Erlenmeyer flasks containing 100 mL of medium and the flasks were incubated for up to six days, at 70°C and at an agitation rate of 200 rpm. Each 24 h, samples were taken and centrifuged (10000 x g, 15 min, 20°C) for the determination of extracellular amylolytic activity, and for determination of the starch and reducing sugar concentrations in the supernatant fluids. For the pupose of evaluating the effect of temperature and medium composition on potential starch degradation, a negative control test was carried out containg the same culture medium without inoculation, incubated at 70°C, with stirring at 200 rpm.

3.2.3. Extraction of amylase activity from the cells

To verify whether or not the amylolytic activity was associated with the cells, cultures were harvested by centrifugation (5000 x g, 5 min) and the cells washed with acetate buffer, pH 5.5 (80 mM). Extraction of the amylolytic activity from cells was carried out by adding a solution of 1% (v/v) Triton X-100 in 80 mM acetate buffer, pH 5.5, using the following ratio: 4 mL of the Triton X-100 solution per 1 g of wet cells. The mixtures were then incubated for 30 min at 70°C at an agitation rate of 200 rpm. After centrifugation (10000 x g, 10 min), the amylolytic activity was determined on the amylase-enriched supernatant fluid samples.

3.2.4. Correction for evaporation during incubation

Evaporation was noticed during culture incubation, due to the high temperature used, and it was essential to add 10 mL of sterile distilled water every 48 h to maintain more or less the culture volume. All culture experiments were replicated to test for reproducibility, and samples were analysed in triplicate. The values shown in this report correspond to mean values with a standard error lower than 10 %.

3.2.5. Optimization of the production of extracellular amylolytic activity by *T. thermophilus* HB8

The optimization phase was conducted in three main steps: (1) by increasing cell density at inoculation time; (2) by screening of the significant parameters influencing extracellular amylolytic activity accumulation using a fractional factorial design with two levels of each factor, and (3) by optimization of the response using a response surface methodology approach.

3.2.5.1. Increasing inoculum cell density

Tests were performed in order to increase the optical density (OD) of the pre-culture (inoculum) at inoculation time by testing four different modifications of the DSMZ-74 medium (Table 3.2). Different initial concentrations of yeast extract, tryptone and NaCl were evaluated.

Table 3.2 Influence of culture medium composition on *T. thermophilus* HB8 cell density for inoculum production.

Culture Medium code	Yeast Extract (g/L)	Tryptone (g/L)	NaCl (g/L)	OD at 600 nm after 24 h
DSMZ-74	4	8	2.00	1.6 ± 0.4
1	10	30	1.38	1.7 ± 0.3
2	10	30	1.50	4.3 ± 0.6
3	15	35	1.50	8 ± 1
4	20	30	2.00	3.5 ± 0.9

Inoculation at 70°C, with agitation rate of 200 rpm.

3.2.5.2. Two level- fractional factorial design (2^k FFD)

2^k FFD is a statistically efficient and economical method leading to suitable approximation for the true functional relationship between the response and the set of the independent variables. The approximation function is a first order model (Montgomery, 2012). To evaluate six independent variables, *i.e.* initial concentration of yeast extract, initial

concentration of tryptone, initial concentration of NaCl, initial concentration of corn starch, initial concentration of biomass (inoculum) and incubation temperature, a set of 21 experiments was required (16 runs of FFD and 5 central points), to be carried out in 500 mL-Erlenmeyer flasks, with a 100 mL-volume of culture medium. To apply FFD, it was assumed that the response should be approximately linear over the range of the factor levels chosen. Table 3.3 presents the codes for the six factors proposed by the model (low level = -1, central point = 0 and high level = +1).

The small-scale batch fermentations were carried out at an agitation rate of 200 rpm for a duration of five days. In all cases, extracellular amylolytic activity was monitored before and after addition of Triton X-100 to the culture (3 g of Triton added to 100 mL of broth, incubation for 1 h at 70°C at an agitation rate of 200 rpm). The purpose of the Triton X-100 treatment was to evaluate the possibility of "extracting" amylolytic activity from the cells without any other treatment than a simple centrifugation. The experimental design used is presented in Table 3.4. The Design Expert version 9.0 software was used to analyze the data and to build the model. The analysis of variance (ANOVA) was performed to test the effect of each factor and wether or not the model was significant.

Table 3.3 Levels of the six parameters and the codes used in the model.

Factor	-1*	+1*	Central Point
Yeast extract (g/L)	4	26	15
Tryptone (g/L)	8	42	25
NaCl (g/L)	0.5	2.5	1.5
Starch (g/L)	5	25	15
Intial biomass (% (v/v))	5	20	12.5
Temperature (°C)	60	70	65

* -1 and +1 present, respectively, the low and high level of each factor.

3.2.5.3. Central composite design (CDD)

Twenty five (25) batch fermentation tests were performed in 500 mL-Erlenmeyer flasks, with 100 mL of culture medium, to satisfy a central composite design (CCD) approach. The CCD employed investigated independent parameters selected according to the results of the FFD step. Table 3.4 describes how each factor was varied in the batch tests. Extracellular amylolytic activity was determined on the 5^{th} day of cultivation. The Design Expert v 9.0

software was used to analyze the data and to build the model. The optimal conditions proposed by the model built were tested experimentally to verify predictability of the model.

3.2.6. Basic characterization of the extracellular amylolytic activity

The influence of temperature and pH on the amylolytic activity present in the crude but clarified extracellular fraction was determined using different temperatures (from 40°C to 100°C) and different pH values (from 3.5 to 12.0). Thermal stability was determined by measuring residual enzyme activity using the standard conditions (70°C, 5 min) following pre-incubation of the enzyme for 24 h at 70°C, 80°C, 90°C and 100°C without any added substrate. Furthermore, different initial concentrations of corn starch (from 0.1% to 3.5% (w/v)) were tested to determine the influence of substrate concentration on enzyme activity.

3.2.7. Analytical procedures

Amylolytic activity was assayed using starch as substrate according to Fuwa's colorimetric method after modifications (Shaw *et al.*, 1995). The reaction mixture contained a preheated 0.5 mL-volume of 80 mM sodium acetate buffer at pH 5.5 (or other buffer according to the assay), 0.25 mL of a 0.1% (w/v) starch solution and 0.2 mL of the sample after appropriate dilution. The mixture was incubated at 70°C for 5 min and the reaction was stopped by adding 0.25 mL of 0.1 M HCl and 0.25 mL of iodine reagent (2% (w/v) KI and 0.2% (w/v) I_2). One unit of activity was defined as the amount of enzyme which decreased the absorbance at 690 nm by one unit in 10 min. The same method with some modifications was used to quantify starch concentration in culture media whenever desired. The mixture contained a 1.0 mL-volume of 80 mM sodium acetate buffer at pH 5.5, 0.2 mL of the sample after appropriate dilution, and 0.25 mL of iodine reagent (2% (w/v) KI and 0.2% (w/v) I_2). The absorbance was measured at 690 nm. The calibration curve to determine the concentration of starch was built by using stantard solutions having concetrations of starch from 0.02% to 0.1% (w/v). Quantification of the reducing sugars accumulating in the culture media was performed according to Bernfeled's colorimetric method using the 3,5 dinitrosalcylic acid reagent (Acer *et al.*, 2016). Pullulanase activity was also evaluated by using limit-dextrinase tablets (Megazyme, T-LDZ-200T).

3.3. Results and discussion

3.3.1. Strain HB8 as a producer of amylolytic activity

Using the standard growth conditions (DSMZ-74 medium with 1% corn starch, 70°C, 200 rpm), no significant extracellular amylolytic activity could be measured over six days of incubation, which is opposite to the results reported by Berger *et al.,* (1995). That could be explained by the fact that the two studies used different conditions for growth. Indeed, in the study of Berger *et al.,* (1995), the temperature of growth was 75°C, and the inducers were lactose and galactose.

Growth on starch was, however, quite rapid, as well as starch consumption, and reducing sugar production and accumulation over time was evident (Figure 3.1 and Figure 3.2). Indeed, the action of amylases on starch is known to release reducing sugars, some of them will be used by the microorganism and some of them will accumulate in the culture medium. These observations led us to put forward the hypothesis that the amylolytic activity of strain HB8 might be present at low level in the soluble fraction (supernatant fluid) but not detectable by the analytical method used, or else, the amylolytic activity migh have been cell surface-bound.

To verify this hypothesis, the mild non-ionic detergent Triton X-100 was added to some cultures at a final concentration of 1% (w/v) in the hope of "extracting" some of the potential cell surface-bound amylolytic enzyme. This led to some success: indeed, a low but detectable amylolytic activity was measured in the extracted material, in the order of 0.08 ± 0.02 U/g of wet cell weight. The biomass obtained with this experiment was 8.3 ± 0.9 g of wet cells /L of culture medium, which means the amylolytic activity obtained by using these conditions was 0.6 mU/mL of the culture medium. The fact that several pullulanases have been identified as cell-associated proteins (Doman-Pytka and Bardowski 2004), such as the pullulanase of *Thermus aquaticus* YT1, which was solubilized by Triton X-100 (Plant *et al.*, 1986), encouraged us to make such attempts. In addition, on the basis that *T. thermophilus* HB8 was able to accumulate an extracellular type 1 pullulanase activity (Tomiyasu *et al.,* 2001), we also attempted to extract a potential cell-surface bound pullulanase from some culture samples. To do so, a pullulanase assay kit (Megazyme, T-LDZ-200T) was used. However, no pullulanase activity could be detected. This observation was, therefore, contrary to the findings of Tomiyasu *et al.*, (2001) and this could be explained by the fact that Tomiyasu *et al.* (2001) introduced several purification and concentration steps before quantifying pullulanase activity (Tomiyasu *et al.*, 2001). Our results, nevertheless, indicated

that the *T. thermophilus* HB8 strain was able to yield low but detectable amylolylytic activity under some process conditions.

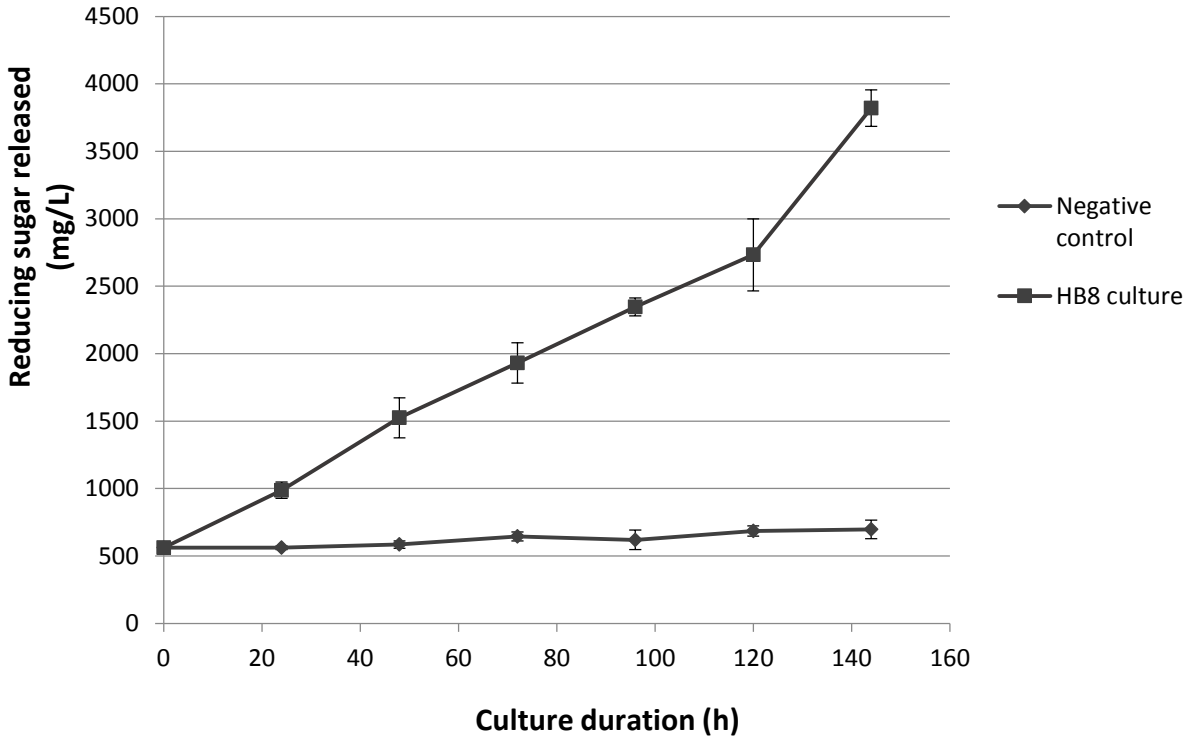

Figure 3.1: Concentration of reducing sugars released from corn starch in culture medium during six days of incubation at 70°C, with stirring at 200 rpm. HB8 culture: fermentation of *T. thermophilus* HB8 growing in DSMZ-74 and 1% (w/v) of starch; Negative control: culture medium DSMZ-74 and 1% (w/v) of starch without inoculation with *T. thermophilus* HB8 (bars represent the standard deviation of the mean).

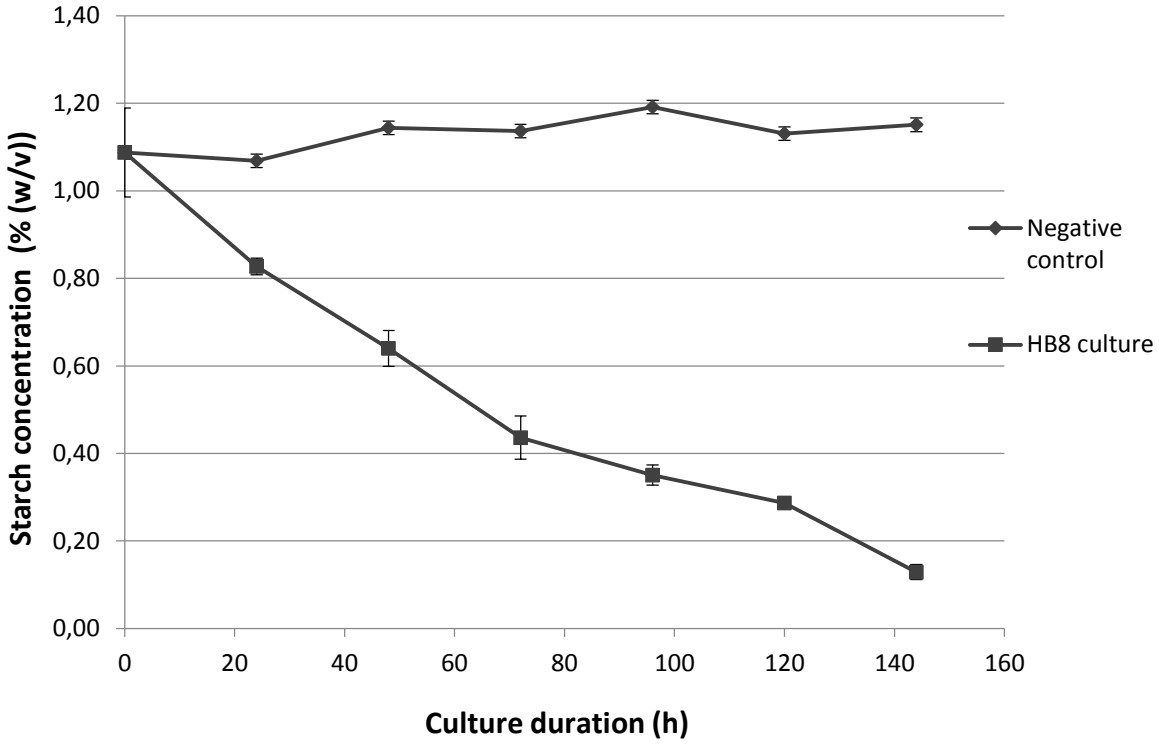

Figure 3.2: Concentration of starch in culture medium during six days of incubation at 70°C, with stirring at 200 rpm. HB8 culture: fermentation of *T. thermophilus* HB8 growing in DSMZ-74 and 1% (w/v) of starch; Negative control: culture medium DSMZ-74 and 1% (w/v) of starch without inoculation with *T. thermophilus* HB8 (bars represent the standard deviation of the mean).

3.3.2. Optimization of extracellular amylolytic accumulation by strain HB8

3.3.2.1. Increasing inoculum cell density

Thermophilic and extreme thermophilic wild-type microorganisms are characterized by low cell density production, and by consequently low enzyme titers (Gupta *et al.*, 2014). For this reason, an increase in inoculum cell density was first required in order to accelerate and possibly increase enzyme production. Using the standard growth conditions (DSMZ-74 medium, 70°C, 200 rpm, 24 h), inoculum cell density was rather low (1.6 ± 0.4 OD at 600 nm). Increasing yeast extract and tryptone concentrations and lowering somewhat the NaCl concentration proved to be very beneficial (Table 3.2). Modification 3 of the DSMZ-74 medium appeared the most promising one, leading to a minimum of a 4-fold increase in cell density. This new medium recipe was, therefore, adopted for the preparation of all subsequent inoculum, unless indicated otherwise.

3.3.2.2. Two level-fractional factorial design (2^k FFD)

FFD was employed to determine the effect of six factors on amylolytic activity production before and after addition of Triton X-100 (3% (w/v)). It is important to notice that, before the FFD was carried out, assays were conducted to choose the most effective concentration of Triton X-100 for extracting amylolytic activity from the cells. Different final concentrations of Triton X-100, from 0.2 to 5% (w/v), were tested on 5 days-old cells grown on starch. A 3% (w/v) concentration of Triton X-100 proved to be optimal.

The results of a first experiment (Table 3.4) showed the appreciable ability of Triton X-100 to extract amylolytic activity from cells without using any other treatment (Runs 1 to 19). Interestingly, the results from Runs 20 and 21 indicated the presence of a high amylolytic activity in the supernatant fluid even before addition of Triton X-100; actually, adding Triton X-100 to these cultures had very little, if any, impact. Helenius and Simons (1975) reported that the success of solubilisation using detergents, such as Triton X-100, is dependent on many factors, such as pH, temperature and the detergent/organic material ratio. Runs 20 and 21 yielded cell densities at least two-fold superior to those of the other experiments and it is likely that the new detergent/organic material ratio was unfavourable for good extraction. A second hypothesis is that the results could be due to differences in culture medium composition. More work on this phenomenon should be conducted in order to obtain a more definite explanation. Design Expert software, version 9.0, was used to perform the analysis of variance in order to study the effect of the six selected factors on amylolytic activity accumulation on the 5th day of cultivation, before Triton X-100 treatment. For the purpose of normalizing the distribution of standardised residuals (Figure 3.3), square root transformation of data was carried out. Equation 1 was therfore built:

Equation 1 in terms of the coded factors:

$$\text{Sqrt (Amylolytic activity+0.06)} = 1.84 + 0.98*YE + 1.09*Tr + 0.65*N + 0.65*I + 0.69*YE*Tr + 0.42*YE*N + 0.91*YE*I$$

The analysis of variance (ANOVA) shows that yeast extract (YE), tryptone (Tr), NaCl (N) and inoculum size (I) all had p-value less than 5%, which meant that they had significant positive effects (Table 3.5). Temperature and starch concentration had p-value more than 5%, which meant no significant effect. The model was significant but the lack of fit of the model was also significant, which meant that the prediction of the model to the response was very weak even if the coefficient of determination R^2 was 0.967. That was due mainly to

non-linearity of the response through the interval of the levels of factors. To build a predictive model, it was necessary to apply a second order model and to reduce the size of the region of the parameters. The results allowed us to identify the composition of a promising culture medium for *T. thermophilus* HB8 permitting to accumulate more than 60 U/mL of amylolytic activity without the need for Triton X-100 treatment (Figure 3.4). This culture medium was made up of 26 g/L of yeast extract, 42 g/L of tryptone, 2.5 g/L of NaCl, and 25 g/L of corn starch. Enzyme production started with a 20% (v/v) inoculum, followed by incubation at 70°C at an agitation rate of 200 rpm. In general, industry and applied microbiologists use an inoculum size in the 10-12% (v/v) range to limit the potential transfer of toxic substances to the new culture medium (Crolla and Kennedy, 2001). In this study, a 20% (v/v) inoculum size was optimal and the reason for this phenomenon is still unclear.

Table 3.4 Concentration combinations of medium components and temperature proposed by the 2^{6-2} FFD with 5 central points, and amylolytic activity accumulation in each run at the 5th day of cultivation, before and after Triton X-100 treatment.

Run	Yeast Extract (g/L)	Tryptone (g/L)	NaCl (g/L)	Starch (g/L)	Inoculum (%(v/v))	Temperature (°C)	Amylolytic Activity Before Triton Addition (U/mL)	Amylolytic After Triton Addition (U/mL)
1	4	8	0.5	5	5	60	0.60	7.80
2	4	8	0.5	25	5	70	0.00	3.70
3	4	8	2.5	5	20	70	0.45	14.12
4	4	8	2.5	25	20	60	0.00	5.50
5	4	42	0.5	5	20	70	1,82	17.90
6	4	42	0.5	25	20	60	0.00	8.03
7	4	42	2.5	5	5	60	2.63	8.17
8	4	42	2.5	25	5	70	3.79	16.12
9	15	25	1.5	15	12.5	65	2.74	13.14
10	15	25	1.5	15	12.5	65	2.91	13.36
11	15	25	1.5	15	12.5	65	2.55	13.58
12	15	25	1.5	15	12.5	65	2.86	14.48
13	15	25	1.5	15	12.5	65	2.76	14.16
14	26	8	0.5	5	20	60	3.28	18.70
15	26	8	0.5	25	20	70	1.64	13.34
16	26	8	2.5	5	5	70	0.79	14.80
17	26	8	2.5	25	5	60	0.00	7.77
18	26	42	0.5	5	5	70	3.40	15.62
19	26	42	0.5	25	5	60	4.60	7.84
20	26	42	2.5	5	20	60	43.70	47.56
21	26	42	2.5	25	20	70	63.18	63.62

Table 3.5 ANOVA for selected factorial model.

Source	Sum of Squares	df*	Mean Square	F Value*	p-value* Prob > F	
Model	71.79	7	10.26	54.51	< 0.0001	significant
A-Yeast extract	15.27	1	15.27	81.18	< 0.0001	
B-Tryptone	19.08	1	19.08	101.39	< 0.0001	
C-NaCl	6.85	1	6.85	36.38	< 0.0001	
E-Inoculum	6.80	1	6.80	36.17	< 0.0001	
AB	7.69	1	7.69	40.86	< 0.0001	
AC	2.81	1	2.81	14.94	0.0022	
AE	13.29	1	13.29	70.66	< 0.0001	
Curvature	0.16	1	0.16	0.84	0.3763	
Residual	2.26	12	0.19			
Lack of Fit	2.25	8	0.28	160.77	< 0.0001	significant
Pure Error	7.000E-003	4	1.750E-003			
Cor Total	74.21	20				

* df = degrees of freedom; F-value = ratio of mean square of the parameter (model, factor, interaction between factors, and curvature of the model) and the mean square of residual; p-value = the probability that the effect of the parameter is significant. The parameter is considered significant when its p-value is less than 5% (confidence interval choosing is 95%).

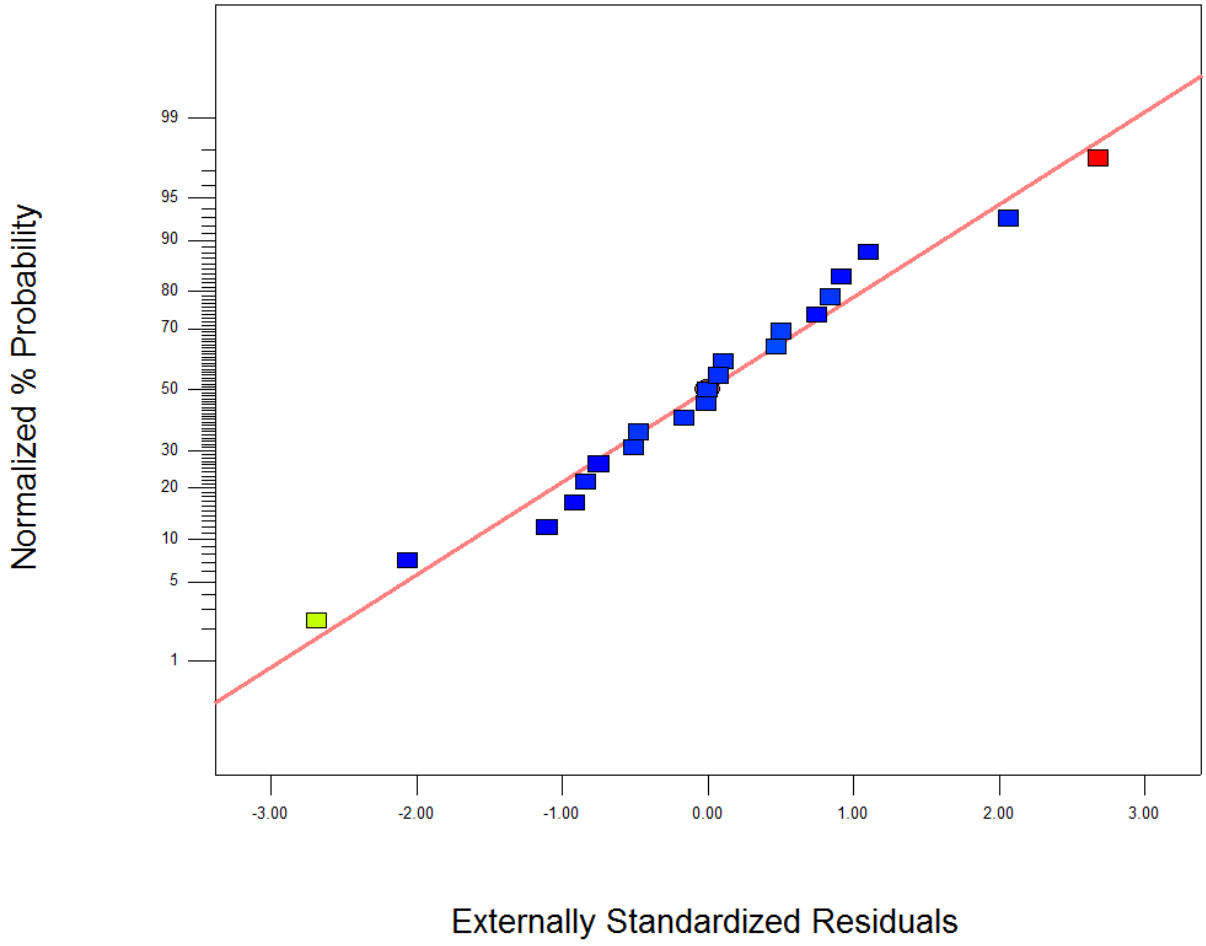

Figure 3.3: Normalized plot of the residuals of the FFD. The normalized plot allows to verify that the data are normally distributed. The red line shows the normal distribution (how the data must be linear) while the red, green, and blue squares represent, respectively, the high, the low and the other probability of the amylolytic activity response.

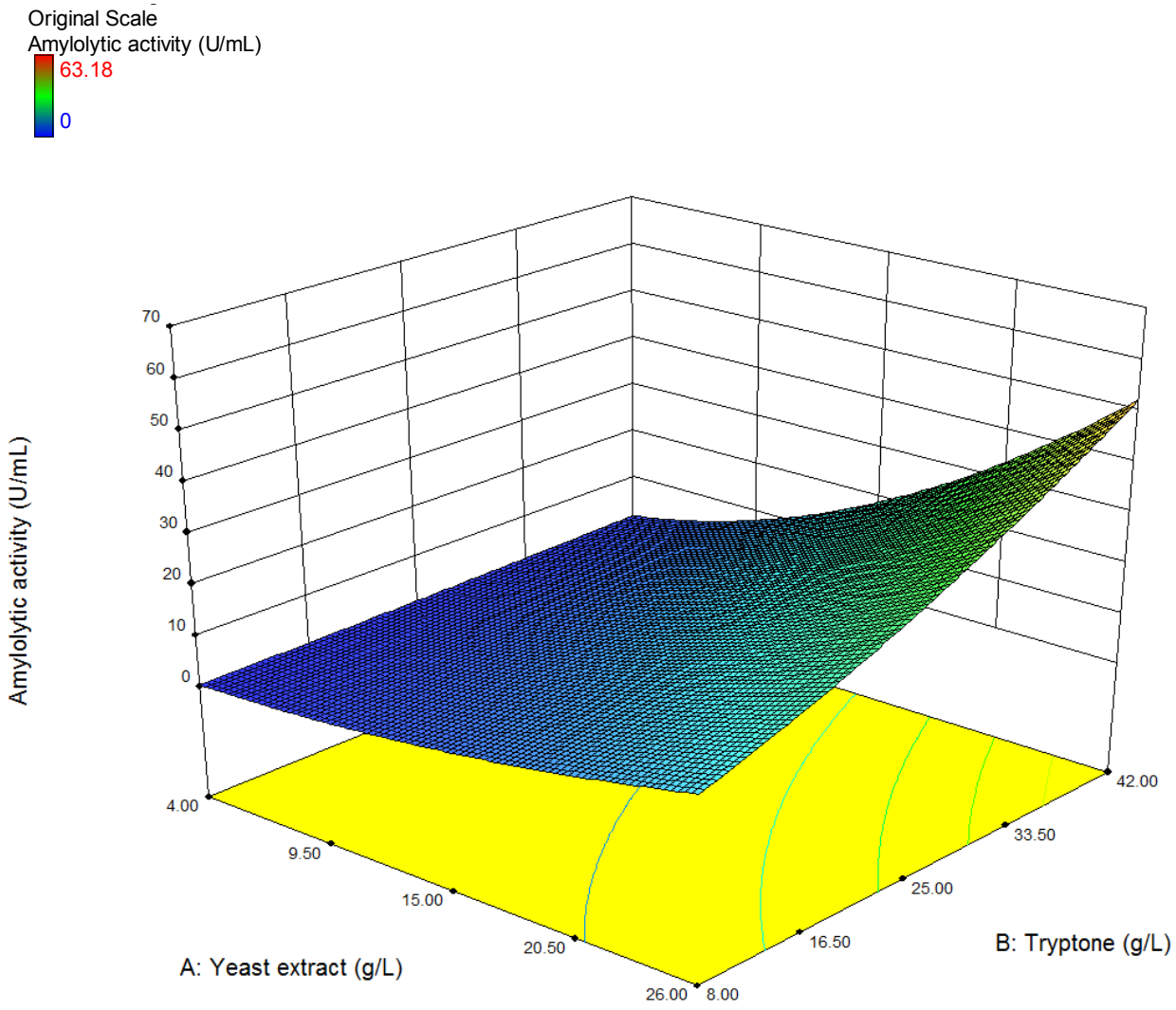

Figure 3.4: 3D surface of the amylolytic activity produced by *T. thermophilus* HB8 strain according to the model built from FFD. Strong interaction effect observed between yeast extract concentration and Tryptone concentration.

3.3.2.3. Central composite design (CCD)

The fermentation factors affecting amylolytic activity accumulation were optimized using a Central Composite Design approach which is a Response Surface Methodology strategy. Three independent variables, namely, initial concentrations of yeast extract, tryptone and NaCl, were included in the model. Initial starch concentration (2.5% (w/v)), inoculum size (20% (v/v)), temperature (68°C) and agitation rate (200 rpm) remained constant during all of the batch fermentation tests. To generate the model, some criteria were taken into consideration: 1/ the level codes (± alpha) were equal to ± 1, in order to increase the number of levels for each factor, and 2/ lack of fit points were increased to eight runs to allow for better predictability of the model. Table 3.6 shows the design matrix of the variables together with 25 runs and the corresponding experimental results for amylolytic activity accumulation in the supernatant fluid after 5 days of fermentation. The analysis of variance showed that the model was significant and that the lack of fit was not significant (Table 3.7), which meant that the model could predict correctly the response with a 95% confidence interval. The response surface plot (Figure 3.5) for amylolytic activity accumulation in the supernatant fluid as function of initial concentration of yeast extract and tryptone showed a strong interaction between the two first parameters. Figure 3.5 shows the strong concomitance between the experimental and predictable responses, except for six (06) points, but at low variance (less than 10%). The model offered an empirical equation for amylolytic activity accumulation as function of yeast extract and tryptone, as well as showing the interaction between yeast extract and NaCl (Equation 2).Equation 2 in term of coded factors (R^2 = 0.754):

Amylolytic Activity Accumulation = 59.93 + 4.46*YE - 2.31*Tr - 7.50*YE*Tr - 4.61*YE*N - 6.30*Tr^2

The theoretical optimal point for amylolytic activity accumulation by the HB8 strain, as defined by the model, was obtained when the culture medium was composed of 40 g/L of yeast extract, 41 g/L of tryptone, 2.0 g/L of NaCl, and 25 g/L of corn starch, combined with a 20% (v/v) inoculum size, a temperature of 68°C and an agitation rate of 200 rpm. The model predicted 68 U/mL of amylolytic activity at this optimal point. To verify the predictability of the model, testing of the optimal conditions in triplicate was carried out. The amylolytic activity produced during 5 days of fermentation was measured. Indeed, no detectable amylolytic activity was found at 24 h of fermentation, 1.77 ± 0.13 U/mL, 14.1 ± 0.24 U/mL, 25.03 ± 2.84 U/mL, and 76.67 ± 4.09 U/mL, respectively at 48 h, 72 h, 96 h,

and 120 h. It would have been interesting to follow the evolution of biomass during the fermentation. However, the culture medium was not homogenous, due to significant cloudiness of the medium caused by the presence of starch, which would interfere with optical density determination. Because cultivation was done for 5 days, cell lysis may have contributed to the release of some amylolytic activity in the supernatant fluids but only to some extent. Since all microbial cultures are heterogeneous, they always contain both young and old cells. Experimentally, the activity obtained was 76 ± 4 U/mL at the 5th of fermentation, which meant the model predicted the response with an 8% deviation, which is quite acceptable.

It was interesting to compare the production results obtained with *T. thermophilus* HB8 using the newly optimized conditions with those obtained with the alkaliphilic *Bacillus halodurans* ATCC 21591 strain, a strain reported to be a good amylase producer (Takami and Horikoshi, 2000). We have tested *Bacillus halodurans* ATCC 21591 and four other *Bacillus* species for extracellular amylase accumulation: *B. halodurans* ATCC 21591 was the best one among the five strains evaluated. Table 3.8 shows conclusively that *T. thermophilus* HB8 offers high potential as an industrially attractive cell factory for the production of a thermostable, extracellular amylolytic enzyme.

Table 3.6 Central Composite Design for three independent variables: initial concentrations of yeast extract, tryptone, and NaCl.

Run	Yeast Extract (g/L)	Tryptone (g/L)	NaCl (g/L)	Amylolytic Activity (U/mL)
1	40.00	41.17	2.00	76.7
2	27.25	56.06	2.40	48.3
3	32.50	44.50	2.40	60.2
4	32.50	44.50	2.40	51.7
5	36.18	43.12	2.80	71.5
6	25.00	44.50	2.66	65.5
7	32.50	56.10	2.72	50.0
8	40.00	30.00	2.80	61.5
9	31.75	50.30	2.00	59.7
10	40.00	30.00	2.30	58.2
11	32.50	30.00	2.00	43.4
12	25.00	59.00	2.80	40.4
13	32.50	44.50	2.40	67.0
14	25.00	40.88	2.00	47.3
15	25.00	30.00	2.80	46.5
16	34.90	59.00	2.32	41.7
17	40.00	48.42	2.46	54.1
18	40.00	59.00	2.00	43.1
19	40.00	59.00	2.80	34.7
20	32.50	30.00	2.00	34.9
21	36.18	43.12	2.80	45.2
22	32.50	44.50	2.40	54.2
23	25.00	59.00	2.00	44.9
24	32.50	30.00	2.60	46.4
25	25.00	30.00	2.30	30.3

Table 3.7 ANOVA for selected central composite design.

Source	Sum of Squares	df*	Mean Square	F Value*	p-value* Prob > F	
Model	2278.02	5	455.60	8.52	0.0002	significant
A-Yeast extract	257.69	1	257.69	4.82	0.0408	
B-Tryptone	72.45	1	72.45	1.35	0.2588	
AB	476.34	1	476.34	8.91	0.0076	
AC	188.00	1	188.00	3.52	0.0762	
B^2	1422.66	1	1422.66	26.60	< 0.0001	
Residual	1016.03	19	53.48			
Lack of Fit	494.05	14	35.29	0.34	0.9500	not significant
Pure Error	521.98	5	104.40			
Cor Total	3294.05	24				

* df is degrees of freedom; F value is ratio of mean square of the parameter (model, factor, interaction between factors, and curvature of the model) and the mean square of residual; p-value is the probability that the effect of the parameter is not significant. The parameter is considered significant when its p-value is less than 5% (confidence interval is 95%).

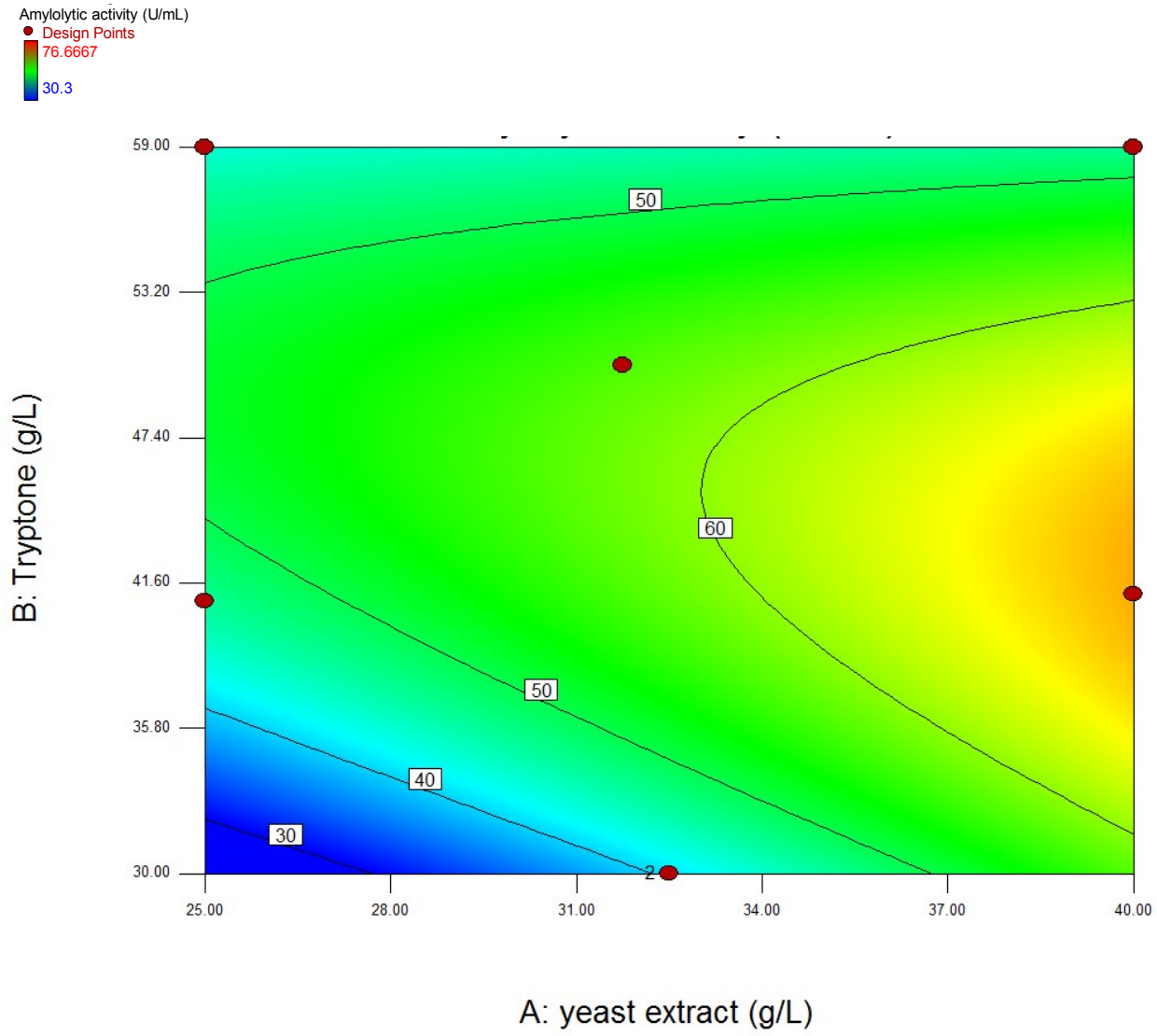

Figure 3.5: Contour plot of amylolytic activity accumulation as function of initial concentrations of yeast extract and tryptone based on the Central Composite Design experimental results. This contour plot characterizes the shape of the surface and locates the optimal amylolytic activity response. The optimal activity is obtained when the culture medium is composed of YE at concentrations in the interval 34 to 40 g/L, tryptone at concentrations in the interval 30 to 53 g/L, and NaCl at 2.0 g/L. The 6 red circles represent design points (experimental responses) which are above the predictable response by the model with a variance less than 10%.

Table 3.8 Comparison of amylolytic activity produced by *T. thermophilus* HB8, initially and after using the newly optimized conditions, with that obtained with *Bacillus halodurans* ATCC 21591.

		Extracellular Amylolytic Activity
Thermus thermophilus HB8	Initial conditions	0.6 mU/mL
	Optimized conditions	76 U/mL
Bacillus halodurans (ATCC 21591)*		1.61 U/mL

* *Bacillus halodurans* ATCC 21591 was grown at 37°C in a 35L-fermenter (airlift design) using soluble starch as main substrate and an initial pH of 10.5.

3.3.3. Effect of temperature, pH and starch concentration on amylolytic activity

The effect of incubation temperature and pH on the amylolytic activity produced by *T. thermophilus* HB8 present in a crude extracellular fraction was evaluated. Amylolytic activity increased sharply from 40°C to 80°C and, thereafter, decreased until the temperature reached 100°C (Figure 3.6). However, the enzyme was still very active at 100°C. The amylolytic activity determined at 80°C and pH 5.5 was 90 ± 2 U/mL. Amylolytic activity was also assayed at different pH values using various buffers: 80 mM sodium acetate (pH 3.5-5.5), potassium phosphate (pH 6.0-7.5), Tris-HCl (pH 7.5-10.0), or Tris-NaOH (pH 11.0-12.0) (Figure 3.7). Amylolytic activity did not really vary between pH 5.0 and pH 7.5 and, then, it increased until pH 9.0. Above pH 10.0, the activity decreased sharply. The fact that the amylolytic preparation was active over a wide pH range, from acidic to alkaline, implies that it can be used in many processes that are subjected to different pH ranges, principally in new biodegradable detergent formulations for applications in biological cleaning and in the textile industry. The pH-activity profile of an amylase depends on the kinetic conformational change of the enzyme, in terms of the optical rotation change through the protonation or deprotonation of amino acid residues of the catalytic site (Horomi *et al.*, 1975). With a pH decrease from 4.0 to 2.0, amylases have shown an alpha-helix uncoiled conformation (Varnavskaia *et al.*, 1978), which can explain the decrease of amylolytic activity. When the enzyme is under alkaline conditions, its catalytic site is more active, which means the pKa values of the catalytic hydrogen donor sites are high (Nielsen *et al.*,

2001). Some authors have reported alkaline-stable amylases, as those isolated from *Anoxybacillus sp.* AH1 (Acer *et al.*, 2016), and from *Bacillus sp.* isolate A3-15 (Arikan, 2008). Therefore, the catalytic site of the amylase(s) produced by *T. thermophilus* HB8 might have the same structure as that of other alkaline-stable amylases (Kolawole *et al.*, 2011).

Regarding thermostability, the crude enzyme was incubated at different temperatures (from 70 to 100°C) for a duration of 24 h, and the residual amylolitic activity was measured under standard conditions (70°C, pH 5.5) (Figure 3.8). The amylolytic activity remained 100% active at 70°C, and 95% active at 80°C, but it decreased sharply at 90°C and 100°C (residual amylolytic activity was less than 10%). The thermostability of amylases has been considered as a requirement for their industrial use in order to maintain high catalytic activity under high temperatures. The most heat-active amylases with an optimal temperature between 90 and 120°C have been identified in species of the genus *Pyrococcus* and in *Methanococcus jannaschii* (Elleuche *et al.*, 2014). The structures of thermoduric amylases showed one or several proline residues located in the surface's loop region (Farhat-Khemakhem *et al.*, 2013). The proline residues reduce the thermal flexibility of the loop, thus, generating high thermostability (Farhat-Khemakhem *et al.*, 2013).

Assays were also performed to evaluate the influence of the initial corn starch concentration on amylolytic activity. Initial starch concentration was varied from 0.1% to 3.5% (w/v). The rate of starch degradation increased more than 5-fold by increasing the initial starch concentration from 0.1 to 2.5% (w/v) and it remained constant thereafter (Figure 3.9).

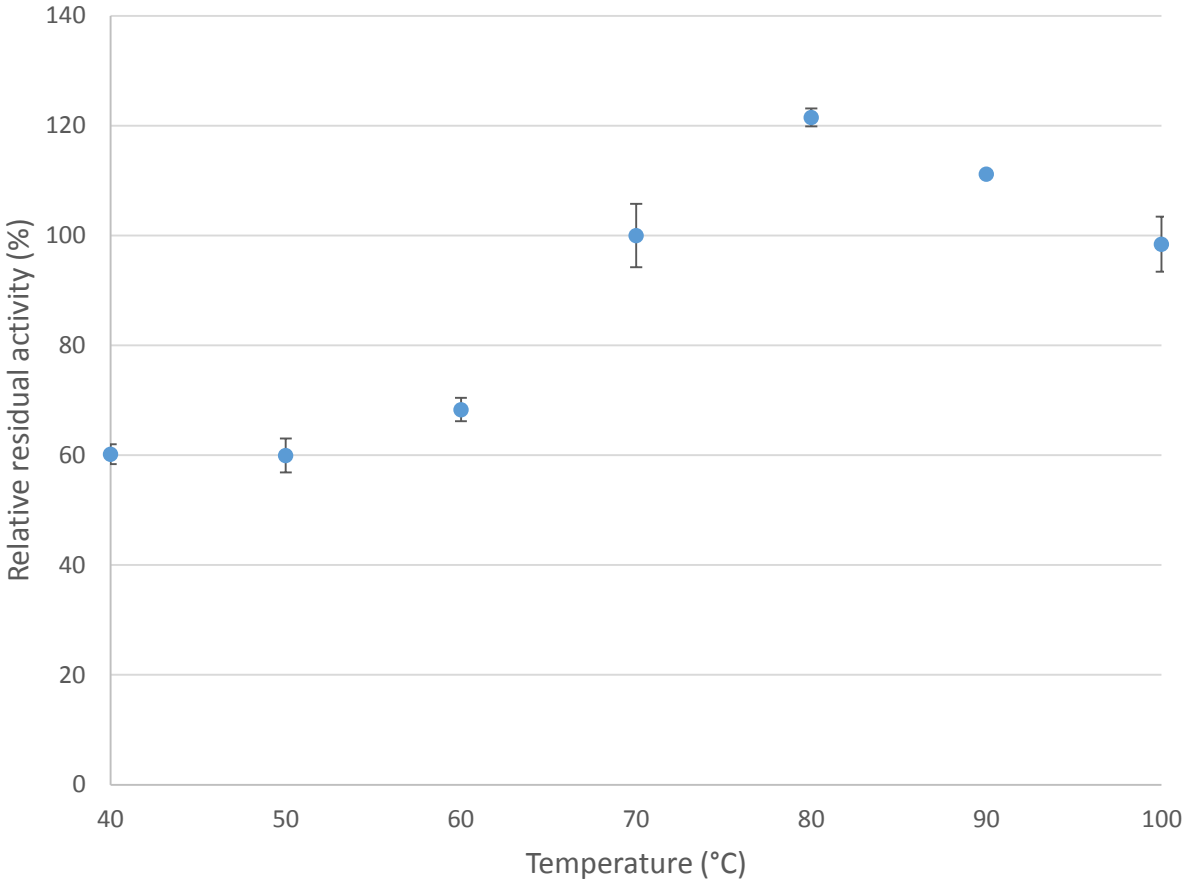

Figure 3.6: Effect of temperature on the activity of the extracellular amylolytic enzyme produced by *T. thermophilus* HB8. Values are shown as percentages of the amylolytic activity observed at temperature tested and pH 5.5. The amylolytic activity observed at 70°C and pH 5.5, was taken as 100% (bars represent the standard deviation of the mean of three assays).

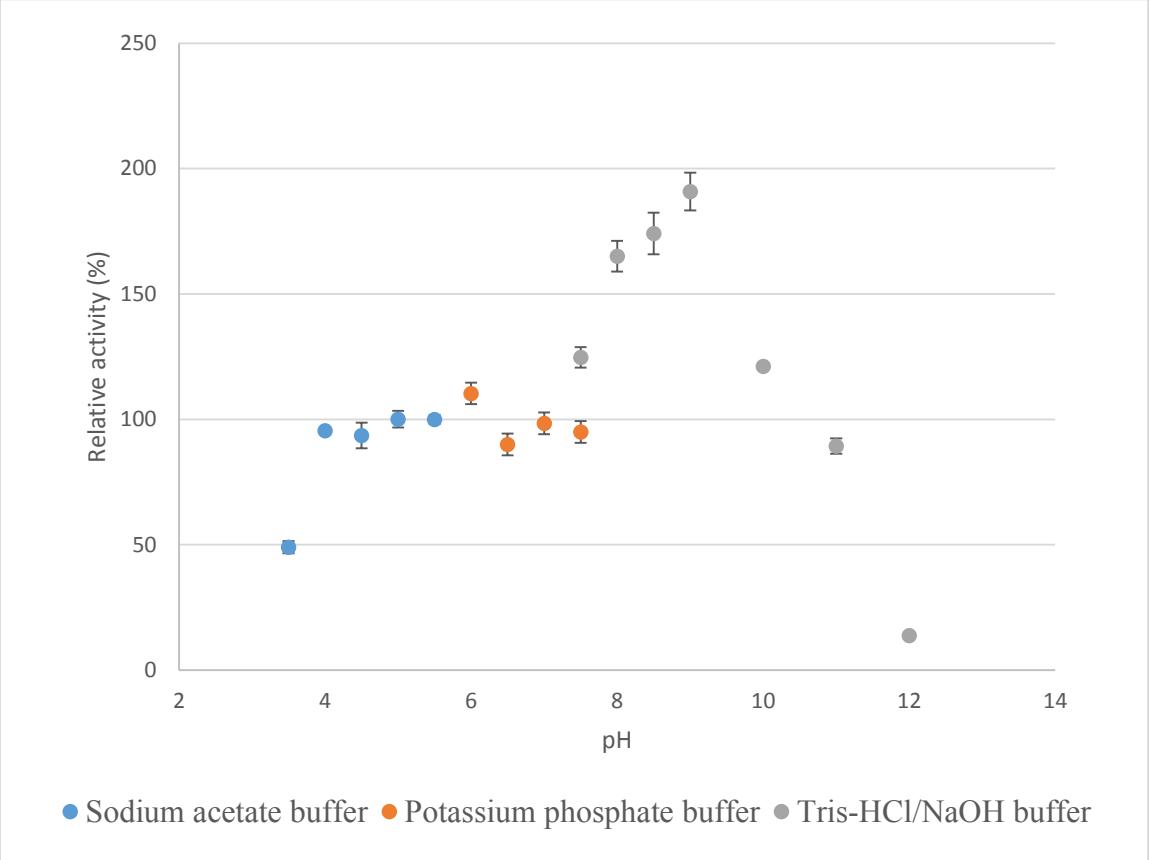

Figure 3.7: pH-activity profile of the extracellular amylolytic enzyme produced by *T. thermophilus* HB8. Values are shown as percentages of the amylolytic activity observed at 70°C and pH tested. The amylolytic activity observed at 70°C and pH 5.5, was taken as 100% (bars represent the standard deviation of the mean of three assays).

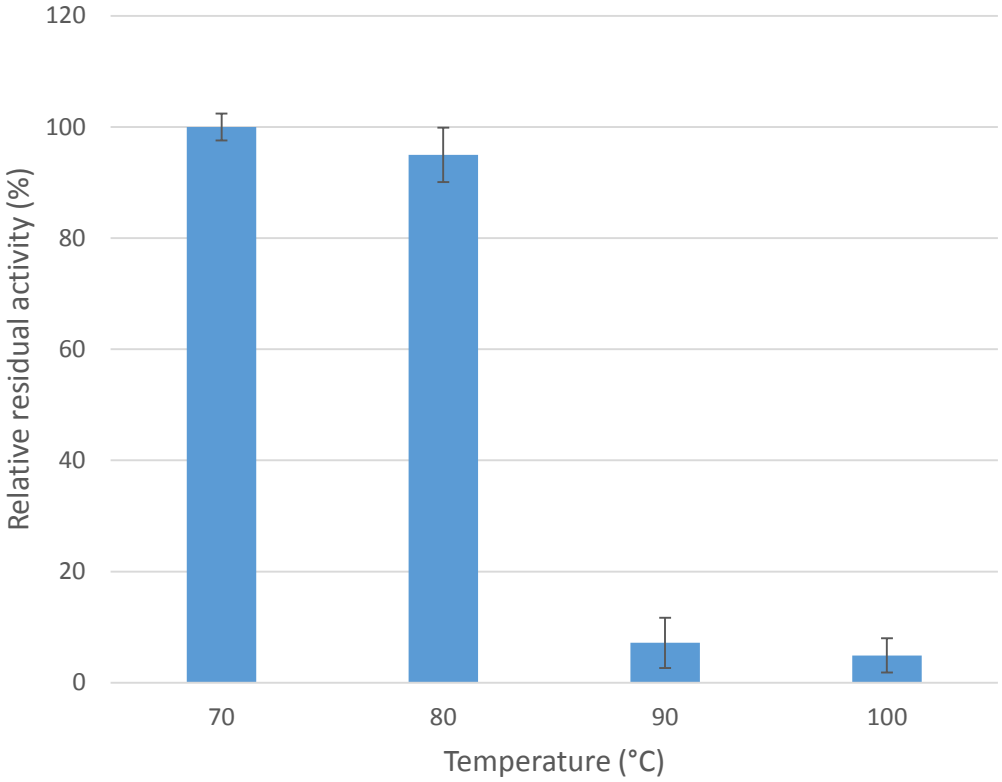

Figure 3.8: Effect of temperature on the stability of the extracellular amylolytic enzyme produced by *T. thermophilus* HB8 after 24 h of incubation at different temperatures. The values are shown as percentages of the amylolytic activity observed at 70°C and pH 5.5, which was taken as 100% (bars represent the standard deviation of the mean of three assays).

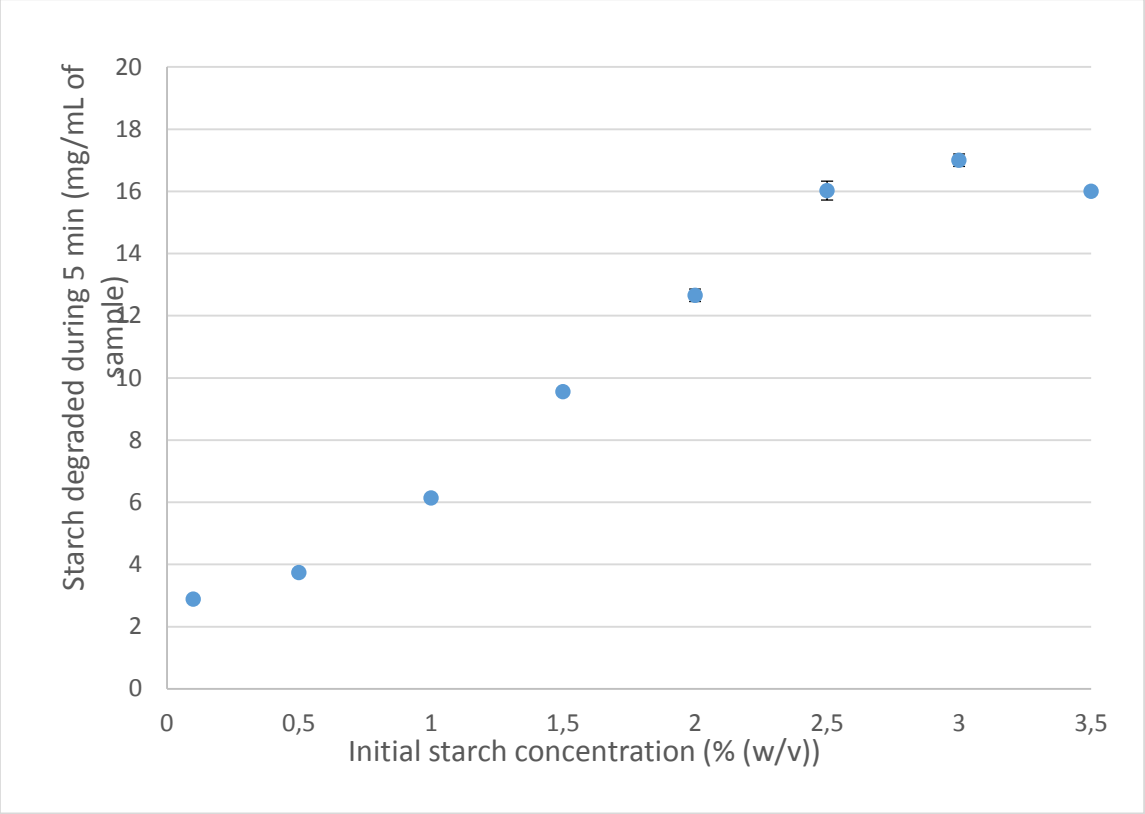

Figure 3.9: Influence of initial starch concentration on the amylolytic activity produced by strain *T. thermophilus* HB8 (bars represent the standard deviation of the mean of three assays).

3.4. Conclusions

The main aim of this work was to evaluate the potential of *T. thermophilus* HB8 to produce and accumulate a significant concentration of extracellular amylolytic enzyme, with the aim of turning *T. thermophilus* HB8 into an industrially attractive source of technical grade, thermostable amylolytic enzyme. Initially, using standard conditions, no significant extracellular amylolytic activity was measured in supernatant fluids. However, applying FFD and CCD strategies during the optimization process proved to be an efficient approach for evaluating the effects of selected factors and for studying the interactions between them. By integrating experimental strategies, mathematical methods and statistical inferences, we have been very successful at converting the process data into empirical equations that predicted the optimal points. Specific conclusions from this work include: (1) A culture medium for *T. thermophilus* HB8 composed of 40 g/L of yeast extract, 41 g/L of tryptone, 2.0 g/L of NaCl, 25 g/L of starch combined with a 20 % (v/v) inoculum size, followed by incubation for 5 days at 68°C at an agitation rate of 200 rpm. Such conditions permitted to obtain an extracellular fraction showing an amylolytic activity of 76 U/mL; (2) the extracellular amylolytic activity produced by *T. thermophilus* HB8, although tested in a rather crude form, was very active and stable at high temperatures and very active at high pH values, which makes this enzyme fraction a good candidate for use under rather harsh process conditions and (3) the production levels obtained with *T. thermophilus* HB8 appear very promising for future industrial applications since they are very competitive with those obtained with several alkaliphilic *Bacillus* species. Work is presently being conducted in order to transfer successfully the present shake flask results to a "bioreactor environment" to permit future technology transfer to industry.

Finally, a recent review of scientific literature seems to indicate that it is very difficult or almost impossible to obtain high enzyme titers with thermophilic or extremophilic microorganisms (Gupta *et al.*, 2014). Our study shows conclusively that a rather classical and systematic approach, combined with patience, can convert a "not promising" microorganism into a "very promising" one. Our results, therefore, offer promises of success with other extremophilic microorganisms.

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Chapitre 4. Production extracellulaire de la pullulanase de *Thermus thermophilus* HB8 par *Pichia pastoris Mut*^{S} et sa caractérisation.

Chapitre 4. Production extracellulaire de la pullulanase de *Thermus thermophilus* HB8 par *Pichia pastoris* Mut^S et sa caractérisation

Avant-propos

Titre de l'article

Paramètres critiques influençant la production extracellulaire de la pullulanase type 1 thermostable *Thermus thermophilus HB8* par *Pichia pastoris* : de la séquence de sécrétion aux conditions environnmentales.

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Le manuscrit présenté dans ce chapitre concerne le deuxième objectif du projet, à savoir l'étude de la capacité de *Pichia pastoris* d'accumuler de façon extracellulaire de la pullulanase type 1 de *Thermus thermophilus* HB8. Aussi, une évaluation de l'effet de plusieurs facteurs sur l'accumulation extracellulaire de cette enzyme fut réalisée.

Résumé :

L'objectif de cette étude était la production extracellulaire de pullulanase thermodurique de *Thermus thermophilus* HB8 par *Pichia pastoris* Mut^S. L'effet de six séquences peptides-signal de sécrétion (SP) sur la production extracellulaire de la pullulanase a été étudié. Deux d'entre eux ayant le SP du facteur alpha de *Saccharomyces cerevisiae* ont montré une accumulation extracellulaire. Cependant, plus de 98% de la pullulanase produite a été accumulée de façon intracellulaire, principalement en raison de la forme dimère de la protéine. Pour améliorer l'accumulation extracellulaire de cette enzyme, le Triton X-100 (TX100) à différentes concentrations et à différents temps d'addition, ainsi que différents osmolytes naturels, différentes valeurs du pH et différentes températures ont été testés pendant la phase d'induction de la fermentation. La production de pullulanase extracellulaire a augmenté de plus de 40 fois en ajoutant de l'acide K-glutamique (0,40% (p/v)) avant 5 h d'induction, du TX100 (2% (v/v)) après 48 h d'induction, et une incubation de 24 h à 30°C et à un pH de 8,0. Les profils d'activité enzymatique en fonction du pH et de température furent aussi établis. La préparation enzymatique a maintenu 50% de son activité après une incubation à 70°C pendant plus de 120 min.

Entête du manuscrit 3

Title

Parameters influencing extracellular production of the thermostable pullulanase of *Thermus thermophilus* HB8 by *Pichia pastoris*: from signal sequence to environmental conditions.

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Abstract

The objective of this study was the extracellular production of the thermoduric pullulanase of *Thermus thermophilus* HB8 by *Pichia pastoris* Mut^S strains. Six secretion signal sequences (SP) were tested. Two of them, possessing the *S. cerevisiae* alpha-factor SP, showed some extracellular enzyme accumulation. However, more than 98% of the pullulanase accumulated intracellularly, due mainly to the dimeric form of the protein. To enhance extracellular accumulation of the enzyme, Triton X-100 (TX-100), used at different concentrations and added at varying times, different natural osmolytes, pH, and temperature were tested during the induction phase of the fermentation. Extracellular pullulanase production increased by more than 40-fold by adding K-glutamic acid (0.40% (w/v)) 5 h before induction, TX-100 (2% (v/v)) after 48 h of induction, and an incubation of 24 h at 30°C and at pH 8.0. The enzyme preparation maintained 50% of its activity during incubation at 70°C for up to 120 min.

Keywords

Thermostable pullulanase, heterologous expression, *Pichia pastoris*, signal peptide sequence, cell permeabilization.

4.1. Introduction

Pullulanases belong to an important group of debranching enzymes, and pullulanases type 1 are usually employed during the starch saccharification step for catalysing the hydrolysis of the α-D-1,6 glucosidic linkages between amylopectin and amylose (Chen *et al.*, 2015; Van Ee *et al.*, 1997). The use of pullulanase(s) in combination with β-amylases increases by 20 to 25% the yield of glucose (Hii *et al.*, 2012b). Also, combination of pullulanases type 1 with a glucoamylase increases by 2% the glucose yield and stops the reverse reaction catalysed by glucoamylases, which means no presence of isomaltose. The isomaltose is a by-product unacceptable in high fructose syrups (Hii *et al.*, 2012b). Pullulanases, in addition, are gaining appreciable interest in other biotechnological industries, for example, in the production of debranched starch, which is used as fat/protein replacer in food products, and in controlled drug release as a tableting excipient (Liu *et al.*, 2017). Finally, pullulanases serve as useful tools for structural studies of carbohydrates (Kunamneni and Singh, 2006), principally those derived from various starches (Hii *et al.*, 2012a). For these reasons, there is a continuing commercial interest in pullulanases.

To date, several microorganisms, including thermophilic and hyperthermophilic ones, have been shown to produce pullulanases, which have been identified and characterized (Gantelet and Duchiron, 1998; Su *et al.*, 2010). However, according to the information available, only one pullulanase preparation is available on the enzyme market for industrial purposes, from the Novozymes Company. The pullulanase gene from *Bacillus acidopullulyticus* is being expressed by *Bacillus licheniformis* and *Bacillus subtilis* (Chen *et al.*, 2014). The enzyme is active and stable at 60°C and pH 5.0 (Domań-Pytka and Bardowski, 2004; Schülein and Højer-Pedersen, 1984).

The heterologous expression approach has been used to produce recombinant thermoduric pullulanases. Most of the time, *Escherichia coli* and *Bacillus subtilis* were used as expression hosts due to their many advantages, mainly their easy availability and expected low production costs (Wang *et al.*, 2014). Construction of the expression vector is a very important step in the success of extracellular production of any recombinant enzyme, thus, selecting the optimal promoter and signal sequence is of critical importance. It was shown, by using *E. coli*, that the T7 promoter is efficient for producing an extracellular pullulanase (Ahmad N. *et al.*, 2014), and extracellular production increased considerably when the T7 promoter was combined with the *lac* operator and the *lacI* gene (Chen *et al.*, 2014). Wang *et al.,* (2014) have observed that using the alkaline protease promoter of *B. alcanophilus*

combined with a levansucrase signal peptide increased extracellular production of the *B. naganoensis* pullulanase by *B. subtilis*.

The methylotrophic yeast *Pichia pastoris* (*Komagataella pastoris*) is one of the more efficient recombinant hosts used in heterogeneous gene expression (Macauley-Patrick *et al.*, 2005). To the best of our knowledge, there are three scientific papers reporting on the production of pullulanases by this yeast. The first one showed the capacity of the phosphatase signal peptide (SP) and that of the α-prepro-peptide of *S. cerevisiae* to increase the extracellular production of an isopullulanase by more than 15 times in comparison to the levels found with the original SP (Akeboshi *et al.*, 2003). The second study reported on an over-expression of the pullulananase from *B. naganoensis* by *P. pastoris,* where extracellular pullullanase production levels in the order of 350 U/ml were obtained (Xu *et al.*, 2006). The third study compared the production of the amylopullunanase of *Geobacillus thermoleovorans* under the control, respectively, of the P_{AOX1} and P_{GAP} promoter (Nisha and Satyanarayana, 2017). They observed that constitutive GAP-based expression led to higher pullulanase production in comparison to inducible AOX1-based expression.

According to Tomiyasu *et al.,* (2001), the *Thermus thermophilus* HB8 strain is one of thermophilic microorganisms showing an ability to produce a thermoduric pullulanase type 1 (80 kDa, 718 amino acids). Despite reported heterologous expression of this gene by *E. coli*, using the *lac* operator, enzyme production was solely intracellular, with very low activity after cell lysis (less than 16 mU/ml of semi-purified enzyme), which was due to the formation of inclusion bodies (Tomiyasu *et al.*, 2001).

For the purpose of over-expressing the thermoduric pullulanase gene from *T. thermophilus* HB8 by *P. pastoris*, for a first time, six SP have been tested to assess their effect on extracellular production/accumulation of the enzyme. In addition, evaluation of the effect of chemical treatments using either Triton X-100 or various natural osmolytes (*i.e.* betaine, proline, guanidine, and potassium glutamate), and of pH and temperature on the secretion of the recombinant pullulanase was performed together with basic characterization of the enzyme. The results of these investigations are reported in this study.

4.2. Materials and methods

4.2.1. Strains and plasmids

Following codon optimization, the pullulanase gene (*Pul*) from *T. thermophilus HB8* (GenBank: BAB62095.1, 718 amino acids, 80 kDa) (Table 4.1) was chemically synthesized by ATUM Inc. and integrated into plasmid pM269. *E. coli* DH5a and *Pichia pastoris* Mut^{s} were, respectively, the cloning and the expression host. Firstly, *E. coli* DH5a containing pM269-*Pul* was grown in selective LB culture medium containing 33 µg/mL of chloramphenicol at 37°C for 18 h. By following the instructions of the supplier (ATUM Inc.), pM269-*Pul* was extracted using the E.Z.N.A® Plasmid mini kit 1 (Omega Biotek, D6942-01), and digested using the IP-Free Electra DAUGHTER™ kit composed of restriction enzyme type IIS (Electra Enzyme *SapI*), to permit integration of the pullulanase gene into six different plasmids (pD912) having the same construction, but with different SP sequences (Table. 4.2). The vector pD912 contained the Alcohol Oxidase gene 1 promoter P_{AOX1} and the Zeocin resistance gene marker. Each plasmid was integrated into *P. pastoris* Mut^{s} by using the electroporation technique and linearized with the restriction endonuclease *SacI*.

Table 4.1: Pullulanase sequence of *Thermus thermophilus* HB8 synthetized and expressed by *Pichia pastoris Mut^{S}*.

MLHISRTFAAYLDEMDQIVVLAPKSLGFDGMAPFTLVAPSGEEIPLSVQHVEDVGETVK YVCRFASAFEFGATYWVRSCRGEETDVQIGAVVRTPAFDDRFFYDGPLGAEYLKEQTV FRVWAPTATAVSVKLVHPHLDEIRCVPLVRGERGVWSAVVPGDWERARYTYIACINRV WREAVDPYATAVSVNGEFGVVIDWEKTKLAPPSLPLPPLCSPTDAIIYELSIRDFTSHPDS GAVHKGKYLGLAETNTSGPNGTATGLSYVKELGVTHVQLMPFMDFAGVDERDPQAAY NWGYNPLHLYAPEGSYATDPADPYARIVELKQAIHTLHENGLRVVMDAVYNHVYDRE QSPLEKLVPGYYFRYDAYGQPANGTGVGNDIASERRMARRWIVDSVVFWAKEYGIDGF RFDLMGVHDIETMKAVRDALDAIDPSILVYGEGWDLPTPLPPEQKATMANAKQLPRFA YFNDRFRDAVKGSTFHLPDRGFALGNPGGREQVKLAIAGSLRALGGLFCHPRQSINYVE CHDNHTFWDKMEAANHDEPEWLRRKRQKLATAIVLLAQGIPFLHSGQEFYRTKGGDG NSYRSPDAVNQLDWERKSRYEDDVRYVQGLIALRRAHGAFRLATEAEVLRHFTFLEPL PPSVIAYRLHDAAVYGPWEDIIVVHHNEEKETAIALPDEREWAVVCDGQRCGTTPFGQA RGMLRLDGIGTWVLVHPAG

4.2.2. Screening of clones

After transformation, the *P. pastoris* clones were cultivated in selective YPDS plates (10 g of yeast extract/L, 20 g of peptone/L, 20 g of dextrose/L, 1.2 M of sorbitol, 20 g/L agar and 250 µg of Zeocin /mL in distilled water). The plates were incubated at 30°C for 36 h. Verification for successful integration of the key genes (*Pul* gene, and Zeocin resistance gene) was carried out for each clone resistant to Zeocin by polymerase chain reaction (PCR) analysis.

The oligonucleotide primers used for PCR amplification were: for *Pul* gene:- Forward: GGATGTTGGAGAGACAGTGAAA,- Reverse: CAGAATCTGGGTGAGAGGTAAAG, and for Zeocin resistance marker gene:- Forward: GCTGTTGAGTTCTGGACTGATA, - Reverse: GCATCACGGAAGTTGGTAGA

The PCR reaction mixture volume was 25 µL, which contained 0.50 µL of forward primer (20 µm), 0.50 µL of reverse primer (20 µM), 0.50 µL of dNTP mixture (10 mM), 2.50 µL of primerStar buffer, 0.25 µL of *Taq* polymerase and 1 µL of yeast lysis extract. The PCR thermocycler conditions involved a denaturation step at 95°C for 5 min, 35 cycles at 95°C for 30 sec, 60°C for 30 sec, 72°C for 2 min, and a final elongation step at 72°C for 5 min. The PCR product was loaded on 1.2% agarose gels, and electrophoresis migration was carried out at 120 V for 30 min.

After confirmation of the *Pul* gene integration, the clones were cultivated in YPD (50 mL) with 250 µg/mL of Zeocin, and the flasks incubated 30°C, at an agitation speed of 200 rpm, for 24 h. The cultures were centrifuged (1000g, 5 min), the supernatant fluid was removed, the cells were suspended in fresh YP medium (YPD without dextrose) and 0.5 ml of pure methanol was added to start induction of *Pul* gene expression. Induction was performed at 30°C, at 200 rpm, for up to five days. Every 24 h, 0.25 mL of pure methanol was added as an inducer and as a carbon and energy source. During induction, both extracellular and cell-associated pullulanase activity was evaluated. Cell lysis was carried out using glass beads (Sigma G8772, 425-600 µm).

The *P. pastoris* clone showing the most promising extracellular pullulanase yield was selected to prepare a Working Cell Bank (WBC) following growth in 50 mL of YPD medium with 250 µg/mL of Zeocin, at 30°C, 200 rpm, for 24 h. To prepare the WCB, 20 mL of a sterile 60% (w/v) glycerol solution was added, to give a final glycerol concentration of roughly 17%. The resulting culture was distributed in sterile tubes (1.5 mL/tube) and stored

at -78 ± 2°C. All subsequent experiments were initiated from this WCB by preparing a pre-culture inoculated by adding 0.8 mL of WCB cells per 100 mL of culture medium containing 250 μg/mL of Zeocin in a 500 mL Erlenmeyer flask using the same operational conditions (30°C, 200 rpm, 24 h).

4.2.3. Enhancement of the extracellular production of pullulanase in shake flask fermentations

In order to permeabilize *P. pastoris* cells for possibly enhancing extracellular pullulanase production/accumulation, Triton X-100 (TX100), various natural osmolytes, and various temperatures during the induction phase were tested during shake flask fermentations employing selected *P. pastoris* Mut^s clones. Firstly, batch fermentations were performed in 500-mL baffled Erlenmeyer flasks containing 100 mL of medium YPD (10 g of yeast extract/L, 20 g of peptone/L, and 20 g of dextrose/L in distilled water), inoculum size was 5%(v/v)), and the flasks were incubated for 48 h, at 30°C, and at an agitation rate of 200 rpm. TX100 was added to final concentration of 2% (v/v) at different times during the induction phase, from 0 to 120 h, followed by 24 h of incubation at 30°C, at the agitation rate of 200 rpm. In parallel, different final concentrations of TX100, from 0.05 to 3% (v/v), were also tested. In a subsequent step, 0.42% (w/v) of a natural osmolyte (i.e. betaine, DL-proline, guanidine, and K- glutamate) was added to the flask fermentation 5 h before the induction phase, followed by treatment with TX100. In addition, different temperatures, from 20°C to 40°C, were tested during induction, using the optimal osmolyte and TX100 concentrations identified earlier together with addition at the optimal time. For each fermentation, both extracellular and cell-associated pullulanase activity was determined, and all assays were performed in triplicate. The data are expressed as the mean ± standard deviation (SD) of these replicates. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was also performed on all supernatants derived from these fermentations.

4.2.4. Improvement in extracellular pullulanase production (bioreactor fermentations)

Batch cultivation was implemented using 3.6 L bioreactors (Labors model, INFORS HT) with a 2 L working volume. pH was maintained by adding automatically 1 M H_2SO_4 or 2 M NaOH. Dissolved oxygen concentration (DO) was maintained at 30% air saturation by cascade adjustment of the agitation rate (from 300 to 1000 rpm) and oxygen supplementation. DO, pH, temperature and agitation rate were recorded using the Iris

Software supplied by INFORS HT. The bioreactor experiments were carried out in two phases: a growth phase and an induction phase.

The growth phase was realised in two stages at 30°C, pH 7.0. During Stage 1, 1600 mL modified YPD medium (40 g of dextrose/L) was used, and the inoculum size was 5% (v/v). Stage 2 began when dextrose had been totally consumed (indicated by a sharp increase in dissolved oxygen level (DO> 80%); at that point, a constant feeding rate of 400 mL YPDc (50 g of yeast extract/L, 100 g of peptone/L, and 200 g/L of dextrose) was implemented at a flow rate of 0.6 mL/min. Once dextrose had been totally consumed, a given natural osmolyte was added to the bioreactor at the final concentration of 0.4% (w/v) and, 5 hours later, pure methanol was added at the constant flow rate of 0.03 mL/min during 5 days. Aeration was turned off when TX100 was added, in order to prevent excessive foaming, and the agitation rate was set at 1000 rpm. Different pH values (4.5 to 8.5), applied during the induction phase, were also tested.

To assess the effect of substrate type during the growth phase on pullulanase gene expression, glycerol was also used in some fermentations, at the same concentration as that of dextrose. Biomass yield and extracellular pullulanase activity obtained during these fermentation experiments were determined. All assays were performed in duplicate. The data are expressed as the mean ± standard deviation (SD) of these replicates.

4.2.5. Characterization of the recombinant pullulanase produced by *P. pastoris*

Firstly, the intracellular pullulanase produced by *P. pastoris* was semi purified by denaturing a fraction of other proteins present in the cell extract following treatment at different temperatures for varying durations and removing the proteins by centrifugation (10,000*g, 5 min). SDS-PAGE analysis permitted to evaluate the purity of the resulting cell extracts. These experiments permitted also to evaluate the thermostability of the pullulanase. The temperature and pH activity profiles of the semi-purified pullulanase were determined, using different temperatures of incubation (from 40°C to 90°C) and different pH values (from 3.5 to 9.0). Furthermore, the effect of different concentrations (50, 95, 140, and 185 mM) of Mg^{2+}, K^{+}, and Ca^{+} ions, and of different concentrations (0.5-2%) of TX100 on the pullulanase activity was investigated. The semi-purified pullulanase was incubated in the presence of each substance during 60 min at 22°C, pH 5.5, and pullulan was then added to determine relative pullulanase activity at 70°C, and pH 5.5.

4.2.6. Analytical methods

Pullulanase activity was determined by measuring the quantity of reducing sugars released from pullulan degradation (Polysciences Inc) during the reaction according to Bernfeld's colorimetric method and using the 3,5 dinitrosalicylic acid (DNS) reagent (Acer *et al.*, 2016). One unit of pullulanase activity was defined as the amount of enzyme which released 1 µmol of reducing sugars (equivalent to maltose) during 1 min. To compare pullulanase production between two fractions assayed (extracellular *versus* cell-associated) all of the pullulanase production values were converted to units of pullulanase activity per original milliliter of the fermentation broth. It was observed that Mg^{2+}, K^{+}, Ca^{+} ions, at some concentrations, interfered with this analytical method by "masking" the presence of reducing sugars. For this reason, pullulanase activity was also evaluated using a special assay kit, implying the use of a limit-dextrinase (Megazyme, T-LDZ-200T).

SDS-PAGE (12% acrylamide) was carried out for determining the molecular mass of the pullulanase. Migration was done using 120 V during 90 min. The gels were then stained with Coomassie Brilliant Blue. To determine the molecular mass of the native pullulanase, native PAGE and zymography tests were carried on by using gels made of 6.5% acrylamide, with and without red-pullulan (Sigma, 0706). Migration was done using 110 V during 2 h. Estimation of the molecular mass was performed by using molecular markers (Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards BIORAD #1610375).

4.2.7. Statistical analysis

All assays were performed at least three times for reproducibility, and samples were analysed in triplicate. The values shown in this report correspond to mean values with a standard error lower than 10 %.

4.3. Results and Discussion

4.3.1. Effect of signal peptide sequence on extracellular pullulanase accumulation

The choice of the signal peptide (SP), located at the N-terminal portion of the protein, is a crucial step, allowing for the protein to cross through the endoplasmic reticulum (ER), where the SP is removed, thus, leading to efficient secretion (Delic *et al.*, 2013). For the first time, the expression vector pD912 was used to express such a heavy protein as the pullulanase (80 kDa) of *T. thermophilus* HB8 by *P. pastoris* Mut^s clone. Six SP sequences were independently integrated into pD912 to evaluate their effect on extracellular accumulation of the pullulanase.

Twenty-six (26) clones resistant to 250 μg of Zeocin/mL were found to carry the *Pul* gene as confirmed by PCR analysis (Table 4.2). Shake flask fermentations of all these clones were carried out to evaluate their capacity to produce extra- and intracellular pullulanase. All of the clones showed intracellular production of pullulanase. However, the clones carrying the vectors pD912-AKS and pD912-AK were able also to produce extracellular pullulanase, as determined on the 5th day of induction, but at low levels, 0.14 and 0.11 U/mL, respectively. However, the possibility that this extracellular enzyme, or a portion of it, could have released due to some cell lysis cannot be neglected. However, even if the SPs AKS, AK and AT share the same amino acid sequence as that of the α-factor of the *S. cerevisiae* pheromone protein (coded in red in Table 4.2), no extracellular pullulanase activity could be detected with clones carrying plasmid pD912-AT. Interestingly, the alpha-factor of *S. cerevisiae* has been previously used to improve extracellular production of a *Geobacillus thermoleovorans* NP33 pullulanase, to give appreciable extracellular enzyme titers (Nisha and Satyanarayana, 2017). However, making a firm comparison between our results and those of Nisha and Satyanarayana (2017) is rather difficult because the latter authors carried out a partial purification of the enzyme before reporting on enzyme titers.

It is important to notice that a "negative control" *P. pastoris* Mut^s strain, which contained plasmid pD912 without the *Pul* gene, was also grown and assayed exactly as the experimental strains; no pullulanase activity could be detected, either intracellularly nor extracellularly.

Table 4.2: List of the different pD912 plasmid constructions tested and type of pullulanase accumulation observed. (Note 1)

pD912 -XX	Signal peptide	Amino acid sequence	Number of clones obtained resistant to 250 µg/ mL of Zeocin in YPDS	Type of pullulanase accumulation
AKS	SP of alpha-factor of *S. cerevisiae* pheromone protein with Kex and cleavage site for protease STE	*MRFPSIFTAVLFA ASSALAAPVNTTTE DELEGDFDVAVLP FSASIAAKEEGVSL EKREAEA	6	Intra- and extra-cellular
AK	SP of alpha-factor of of *S. cerevisiae* pheromone protein with natural cleavage site	*MRFPSIFTAVLFA ASSALAAPVNTTTE DELEGDFDVAVLP FSASIAAKEEGVSL EKR	4	Intra- and extra-cellular
AT	SP of alpha-factor of of *S. cerevisiae* pheromone protein with natural cleavage site	*MRFPSIFTAVLFA ASSALA	5	Intracellular
AA	SP of alpha-amylase gene of *Aspergillus niger*	MVAWWSLFLYGL QVAAPALA	3	Intracellular
KP	SP of toxin killer gene of *S. cerevisiae*	MTKPTQVLVRSVSI LFFITLLHLVVA	4	Intracellular
SA	SP of human albumin gene	MKWVTFISLLFLFS SAYS	4	Intracellular

Note 1: Six (6) signal peptide (SP) sequences integrated into plasmid pD912 to evaluate their effect on the type of pullulanase accumulation (intra- and/or extra-cellular).

Note 2: Low levels of extracellular pullulanase accumulation.

4.3.2. Triton X-100 effect on the release of pullulanase in flask fermentations

One clone of *P. pastoris,* carrying pD912-AKS (Table 4.2), was selected to evaluate the effect of various factors on extracellular pullulanase accumulation. Batch fermentations of this clone were carried out and, during the induction phase, pullulanase activity was measured repeatedly. The majority of the recombinant pullulanase accumulated intracellularly, unfortunately, with a maximal level of 7.32 U/mL on the 5th day of induction (Figure 4.1.A). In parallel, the level of production of extracellular pullulanase was only 0.14 U/mL.

The non-ionic and mild detergent Triton X-100 (TX100) was used to facilitate the release of the recombinant intracellular pullulanase from *E. coli*, due to its capacity to permeabilize

cells (Duan *et al.*, 2015). In a preliminary step, the optimal time for Triton X-100 supplementation and its optimal concentration were investigated. Firstly, 2% (v/v) of Triton X-100 was added at 0, 24, 48, 72, and 96 h during the induction phase. Afterwards, a 24 h-incubation was realized at 30°C, at an agitation rate of 200 rpm, then, pullulanase activity in the "new" supernatant fluid was measured (Figure 4.1.B). Maximal pullulanase activity was observed when TX100 was added at 48 h following induction, with a total production level of 9.51 U/mL (4.64 U/mL of extracellular activity + 4.87 U/mL of intracellular activity), which was almost a two-fold increase compared to the culture without TX100 (total of 5.39 U/ml). After treatment with 2% (v/v) TX100 (final concentration), almost 50% of the pullulanase produced had been released into the culture medium. It has been reported that hyper-thermophilic proteins exhibit significantly reduced hydrophobic accessible surface areas compared with mesophilic proteins (Li *et al.*, 2015). TX100 might indeed increase the solubility of the pullulanase, which would explain the increase in extracellular activity. SDS-PAGE analysis of supernatant fluids derived from presence or absence of TX100 treatment was performed and showed the presence of additional proteins in the treated samples, which would indicate that the TX100 treatment was not really selective.

Using the identified optimal time for TX100 supplementation (48 h after beginning of induction), various final concentrations of TX100, from 0.05 to 3% (v/v)), were tested (Figure 2). Total pullulanase production arose from 4.03 to 7.61 U/mL when the TX100 concentration was increased from 0.05 to 0.1% (v/v). By increasing TX100 concentration from 0.1 to 2% (v/v), extracellular pullulanase production/accumulation was increased by almost three times, from 1.64 to 4.56 U/mL. Pullulanase production remained essentially constant at concentrations superior at 2% (v/v) of TX100. We suggest that the effect of low concentrations of TX100 was due to partial permeabilization of the yeast cytoplasmic membrane. TX100 polar head groups act on the hydrogen bonds present within the lipid bilayer of the cells, thus, leading to the destruction of the integrity of the lipid membrane (Koley and Bard, 2010). However, protein solubilization limits are by no means absolute since micelle formation depends on many factors such as the detergent/solubilizate (cell membrane) ratio, pH and temperature (Helenius and Simons, 1975). In this study, it is possible that TX100 played a dual function when supplied to the culture media. It permeated the *P. pastoris* cytoplasmic membrane and increased the solubility of the recombinant pullulanase. TX100 was successfully used to enhance extracellular pullulanase production

by *Bacillus dermaticans*, and the pullulanase was almost 100% extracellular when 2% of TX100 was added (Duan *et al.*, 2015).

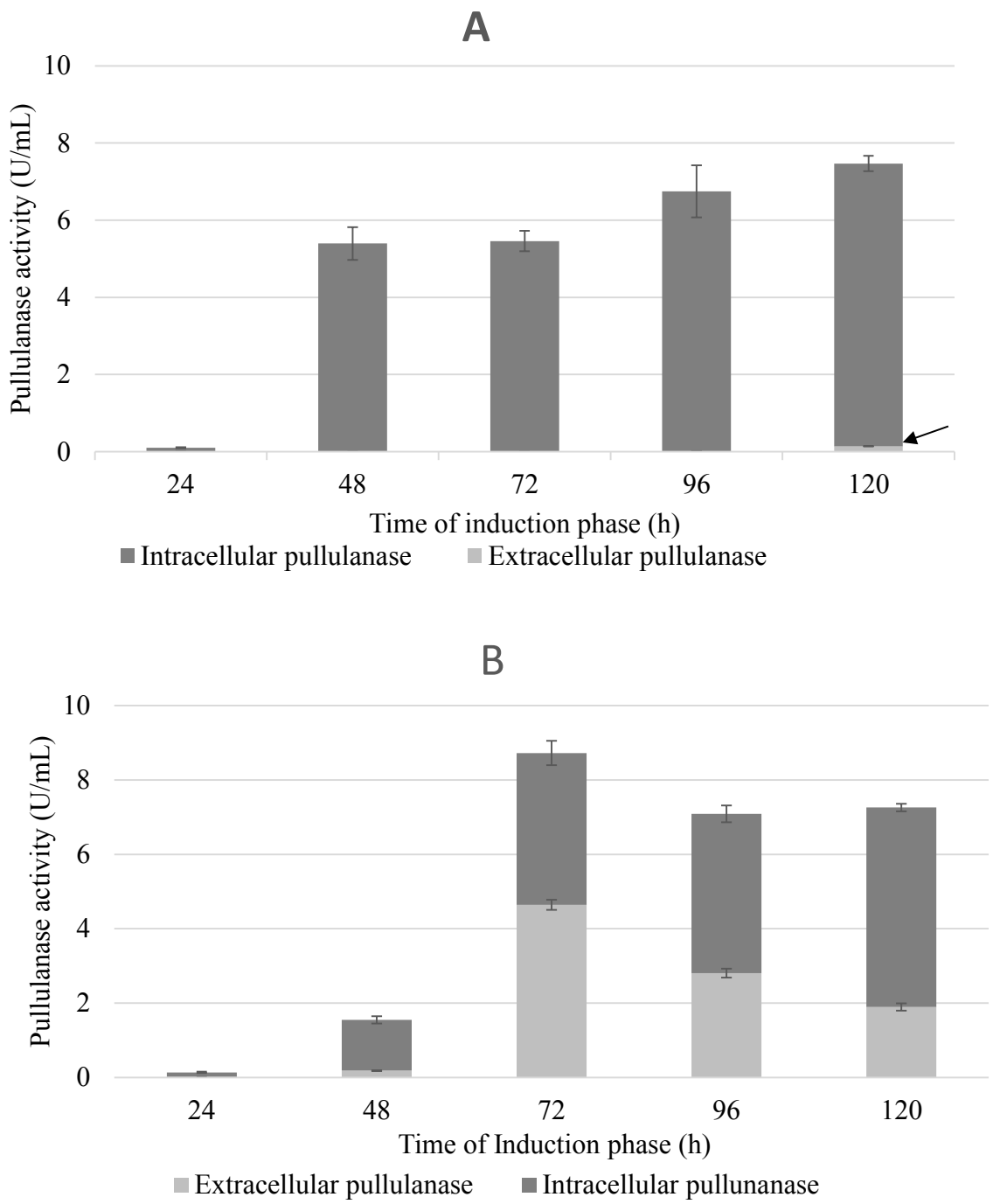

Figure 4.1: Pullulanase activity as a function of duration of induction with the selected *P. pastoris* pD912-AKS-*Pu*l clone. **A**: without any chemical treatment, **B**: when 2% (v/v) of Triton X-100 (final concentration) was added to the culture medium at various times during induction, followed by 24 h of incubation at 30°C, at an agitation rate of 200 rpm (bars represent the standard deviation of the mean).

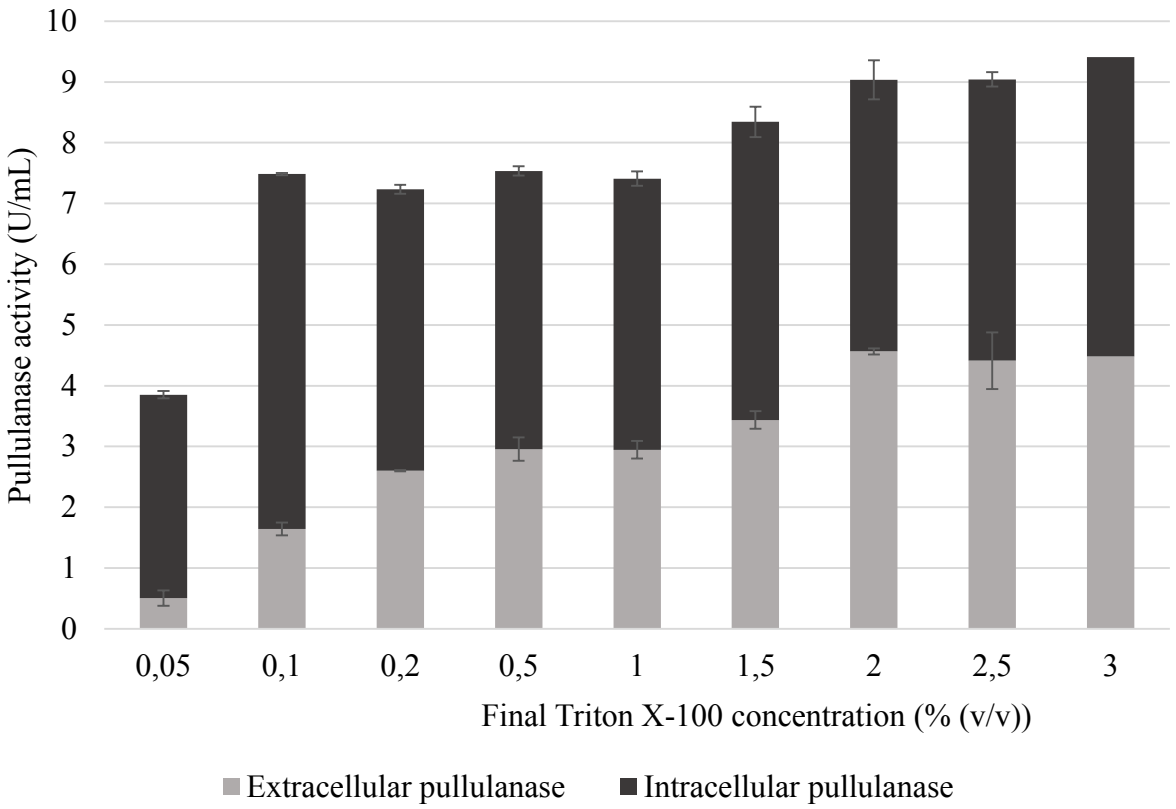

Figure 4.2: Pullulanase activity as a function of final Triton X-100 concentration in cultures. Triton X-100 was added after 48 hours of induction. Enzyme assays were performed 24 hours following induction. Incubation was carried out at 30°C, and at an agitation rate of 200 rpm (bars represent the standard deviation of the mean).

4.3.3. Natural osmolytes and temperature effects on pullulanase excretion in fermentation flasks

It was found that an increase in the concentration of some natural osmolytes can specifically activate CIpB (a protein belonging to the chaperonin disaggregation network) under combined salt- and heat-stresses, resulting in an decrease of inclusion body formation in the case of *E. coli* (Diamant *et al.*, 2003). Duan *et al.,* (2013) have used this feature to evaluate the effect of some natural osmolytes (betaine, proline and K-glutamate) on pullulanase solubilisation produced by *E. coli*. It was shown that 20 mM of betaine, when added two hours prior to induction, led to an increase in periplasmic pullulanase accumulation (45.3 U/mL) but had no effect on extracellular accumulation; however, when betaine was combined with TX100, the extracellular activity increased three-fold.

In this context, four natural osmolytes (proline, guanidine, betaine and K-glutamate) were independently supplied to the culture medium, at a final concentration of 0.4% (w/v), 5 hours before induction. No significant effect on extracellular pullulanase production was observed. However, when TX100 was combined with the natural osmolyte treatments, it was observed that guanidine and betaine led to much inferior total production of the pullulanase, while K-glutamate showed a strong positive effect on pullulanase production. Effectively, the K-glutamate addition, combined with TX100 treatment increased total pullulanase activity from 7.61 U/mL to 10.46 U/mL, of which 6.88 U/mL was extracellular activity (Figure 4.3).

The principal role of the osmolyte is the maintain cell homeostasis under harsh environment and stress condition by their potent influence on protein folding instability. The osmolyte mechanism is universal and independent of primary sequence of the protein. The osmolytes predominately affect stability via their influence on the protein backbone, with sidechains making only minimal contribution. Since most backbone groups in folded protein are not solvent accessible, osmolyte primarily affect stability via changes in the free energy of the unfolded state (Street *et al.*, 2010). In the case of the present study, the osmolyte can interact with the chaperones proteins and/or directly with the pullulanase in its unfolded state (aggregation).

Temperature during induction is also an important parameter for recombinant protein production in *P. pastoris* (Zhang *et al.*, 2007). It was reported that a low temperature facilitates proper folding and, thereby, may improve pullulanase production (Duan *et al.*, 2013). To optimize the temperature for pullulanase production*, P. pastoris* cells expressing pullulanase were induced at different temperatures (20, 25, 30, 35 and 40°C). It was

determined that the optimal temperature was 30°C. When the temperature increased or decreased by 5°C, the extracellular activity decreased by almost 90%.

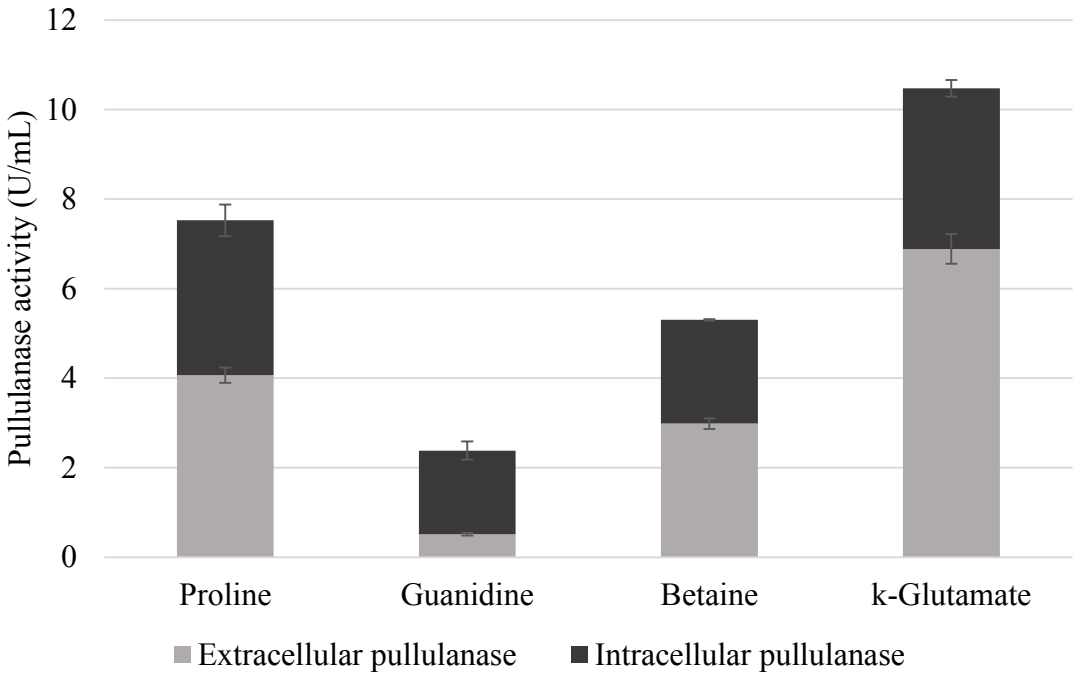

Figure 4.3: Pullulanase activity as function of the osmolyte added at a final concentration of 0.4% (w/v) 5 hours before the start of induction. Addition of Triton X-100 (final concentration of 2% (v/v)) was done 48 h after induction. Incubation was continued for 24 hours at 30°C, at an agitation rate of 200 rpm. The above samples were withdrawn after 72 hours of induction (bars represent the standard deviation of the mean).

4.3.4. pH and type of substrate effects on pullulanase release by *P. pastoris*

In this study, a two-stage dextrose feeding strategy was applied to achieve high-cell-density cultivation of *P. pastoris* (pH was set to 7.0) in the 3.6 L bioreactor. The first stage was a batch fermentation using a modified YPD medium, which achieved a dry cell weight of 50 g/L after 20 h of cultivation. The second-stage began when dextrose had been consumed. During this stage (14 h), the YPDC medium feeding rate was constant (0.6 mL/min), and the dry cell weight reached 73 g/L. K-glutamate was then added at a final concentration of 0.4% (w/v), and after 5 h (transition phase), pure methanol was added at a constant rate of 0.03 mL/min during 5 days to induce and maximize expression of the recombinant gene. Based on earlier observations, TX100 was added 48 h after the start of the induction phase, aeration

was shut down to mitigate foaming and the agitation rate was set at 1000 rpm.

It was also reported that pH has a significant effect not only on protein secretion but also on the micellar properties of non-ionic surfactants and of ionic surfactants with strongly acidic or basic groups, in addition to effects on membrane solubilisation (Helenius and Simons, 1975). The pH determined from flask fermentations, was around 8.3 during the induction phase. For assessing the effect of pH on extracellular pullulanase accumulation, *P. pastoris* cells expressing pullulanase were induced at different pH values, i.e. 4.5, 5.5, 7.0, 8.0, and 8.5. As depicted in Figure 4.4A, a significant effect of pH on extracellular pullulanase accumulation was observed. The optimal pH was 8.0-8.5, which allowed for a high extracellular pullulanase activity of 13.3 U/mL after 36 h of TX100 treatment and the intracellular activity of 6.7 U/mL. Afterwards, the extracellular activity decreased to 10.7 U/mL, this decrease was probably due to either proteolysis activity or pullulanase denaturation under the alkaline conditions. Even if the biomass level was much higher in bioreactor fermentations (76 g of dry cell/L) compared to the levels obtained in shake flasks, the extracellular pullulanase yields obtained in the bioreactor fermentations, when pH was 8.0 (12 U/mL at 36 h), were not much higher than those obtained in shake flask fermentations (8 U/mL at 36 h). This may be explained by the fact the ratio of TX100/biomass was much lower in the first case, probably leading to inferior permeabilization of the cell membrane and decreased solubilisation of the protein. When pH was decreased to 7.0 or 5.5, extracellular pullulanase accumulation decreased to 4.7 and 2.0 U/mL respectively, after 36 h of TX100 treatment. In the case of pH 4.5, no extracellular pullulanase activity was detected during the fermentation. It is important to notice that optical density decreased sharply during TX100 treatment when pH was set above 7.0, but it was almost stable when pH was in the acid range (4.5 to 5.5), which strongly suggests that alkaline conditions may have caused cell lysis and liberate more pullulanase.

Culture medium composition is also an important parameter in industrial protein production. Glycerol as substrate is of particular interest due to its ample availability as by-product of many industrial processes, such as biodiesel production. However, it has been reported that glycerol may repress the expression of genes by *P. pastoris* when still present in the culture medium during induction (Ahmad M. *et al.*, 2014). Using the same feeding strategy described earlier, glycerol was used instead of dextrose to compare its effect on pullulanase production with those determined with dextrose. During the growth phase, the dry cell weight achieved was 50 g/L, and it became 82 g/L after the second stage. As depicted in

Figure 4.4B, pullulanase production were essentially the same, indicating that dextrose and glycerol showed no real difference. A slight increase in extracellular pullulanase activity was observed after 48 h of TX100 treatment, to give 15.47 U/mL of enzyme. The intracellular activities were 6.7 U/mL and 5.2 U/mL in cultures using dextrose and glycerol as carbon source, respectively. Specific extracellular productivity is an important parameter in industrial protein production. Based on the bioreactor fermentations described above, using dextrose or glycerol as substrate during the growth phase, the specific productivity numbers were 186.30 and 188.65 U/g of dry cell weight for dextrose and glycerol, respectively.

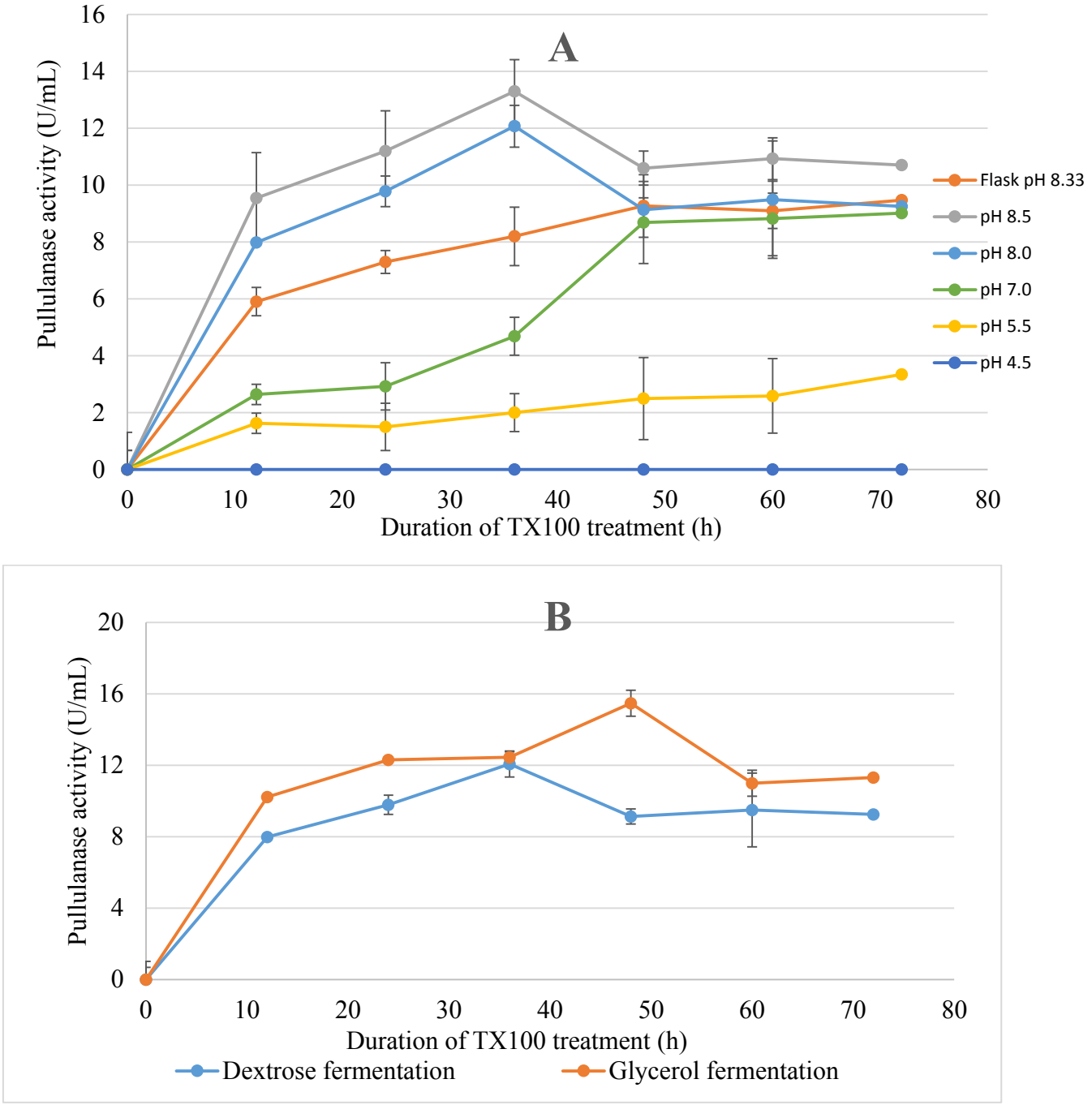

Figure 4.4: Extracellular pullulanase activity produced as function of the duration of the TX100 treatment. The fermentations were conducted in 3.6 L bioreactors. **A**: using dextrose as substrate during the growth phase and induction conducted at various pH values; **B:** using dextrose or glycerol as substrate, with induction conducted at pH 8.0.

4.3.5. Basic characterisation of the recombinant pullulanase

To characterize the recombinant pullulanase produced by the *P. pastoris pD912-AKS-Pul* clone, firstly, a cell extract was incubated at different temperatures (from 40 to 80°C) during different periods (from 15 to 60 min). Pullulanase activity remained almost 100% active at 50°C during 60 min, it decreased to 55% when the enzyme was incubated at 60 - 70°C during 60 min, and it sharply decreased to 5% when incubated at 80°C during 60 min (Figure 4.5A). It is important to notice that 50% of the initial enzyme activity could be recovered after incubation for 3 h at 70°C, therefore, proving the very high thermostability of the enzyme preparation.. SDS-PAGE analysis of cell extracts after thermal treatment, followed by centrifugation to remove some of the native proteins produced by the mesophilic yeast, showed that the thermal treatment could semi-purify the enzyme. Many proteins were removed from the extract when the thermal treatment was applied, mainly at 70°C and 80°C during 15 min, but the pullulanase could still be observed on the SDS-PAGE gels. In order to identify the native form(s) of the pullulanase, semi-purified pullulanase (treated at 60°C during 15 min) was analysed by native PAGE and zymography. Figures 4.5B and 4.5C show clearly that there are two forms of the pullulanase, with molecular masses of 160 kDa, and 80 kDa. The most active form is the dimeric form (160 kDa), the large size of the enzyme possibly the reason why the enzyme was not secreted by *P. pastoris*. Zouari Ayadi *et al.,* (2008) reported that the *B. thermoleovorans* US105 pullulanase, which is 100% similar to that of *T. thermophilus* HB8*,* is active when it is in its dimeric form, which agrees with the result of the present study.

The effects of incubation temperature and pH on the semi-purified pullulanase activity produced by *P. pastoris* were investigated. Enzymatic activity increased sharply when the analysis was carried on at temperatures from 40°C to 70°C. When the incubation temperature increased to 80-90°C, the enzymatic activity decreased. However, the enzyme was still active at 90°C (Figure 4.6A). Pullulanase activity at 90°C and pH 5.5 was 1.7 ± 0.3 U/mL using the semi-purified enzyme. Pullulanase activity was also assayed at various pH values using different buffers (Figure 4.6B). It was observed that pullulanase activity decreased sharply when the pH was acidic (4.0-5.0) or basic (7.5-9.0). However, the enzyme was still active at these “extreme” conditions. The fact that this pullulanase was active over a wide pH range, from acidic to alkaline, imply that it could be used in many processes covering a wide pH range, for instance, in new biodegradable detergent formulations for applications in biological cleaning and in the textile industry.

The effects of Mg^{2+}, K^{+}, and Ca^{+} ions, and of TX100 on the semi-purified pullulanase activity were also investigated. Pullulanase activity was noticeably enhanced by K^{+} at 50 to 185 mM by 20%, decreased by 30% when 185 mM of Ca^{+} ions was added, and no effect of Mg^{2+} was noticed, even at 185 mM. TX100 increased pullulanase activity approximately by 20% when 0.5 to 2% (v/v) of TX100 was included in the reaction mixture, which can suggest that this detergent increases the solubility of the pullulanase.

Assuming that the pullulanase kinetics followed the Michaelis-Menten model, the apparent Km and Vm values of the recombinant pullulanase were calculated as follows: Km = 0.52 % (w/v) pullulan and 1.35 mg of maltose/min, respectively, at 70°C and pH 5.5.

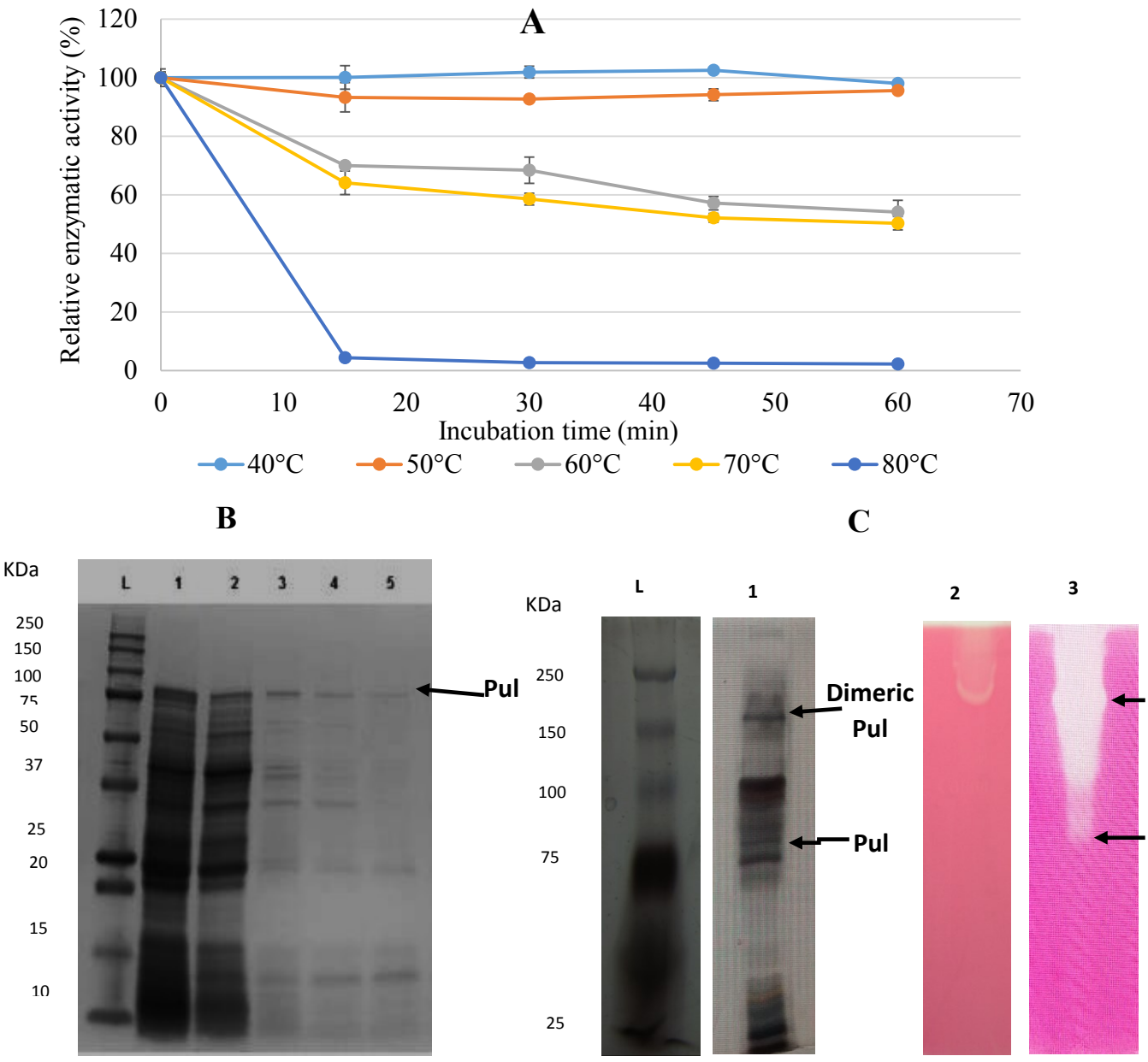

Figure 4.5: Analysis of intracellular pullulanase obtained with the selected *P. pastoris* pD912-AKS-*Pul* clone and effect of thermal treatment on enzyme activity. **A:** Thermostability of the pullulanase incubated at various temperatures. **B**: SDS-PAGE analysis of the intracellular pullulanase fraction before and after thermal treatment for 15 minutes at various temperatures: L = molecular mass markers; 1 = 22°C; 2 = 50°C; 3 = 60°C; 4 = 70°C and 5 = 80°C. **C:** Native PAGE and zymographic analysis of the intracellular pullulanase after thermal treatment at 60°C during 15 minutes. A 6.5% acrylamide gel was used. Migration was carried out at 110 V during 2 h. L = molecular mass markers; 1 = cell extract native proteins revealed by silver nitrate; 2 = zymograph without incubation; 2 = zymograph after 2 h of incubation at 40°C, in acetate buffer pH 5.5.

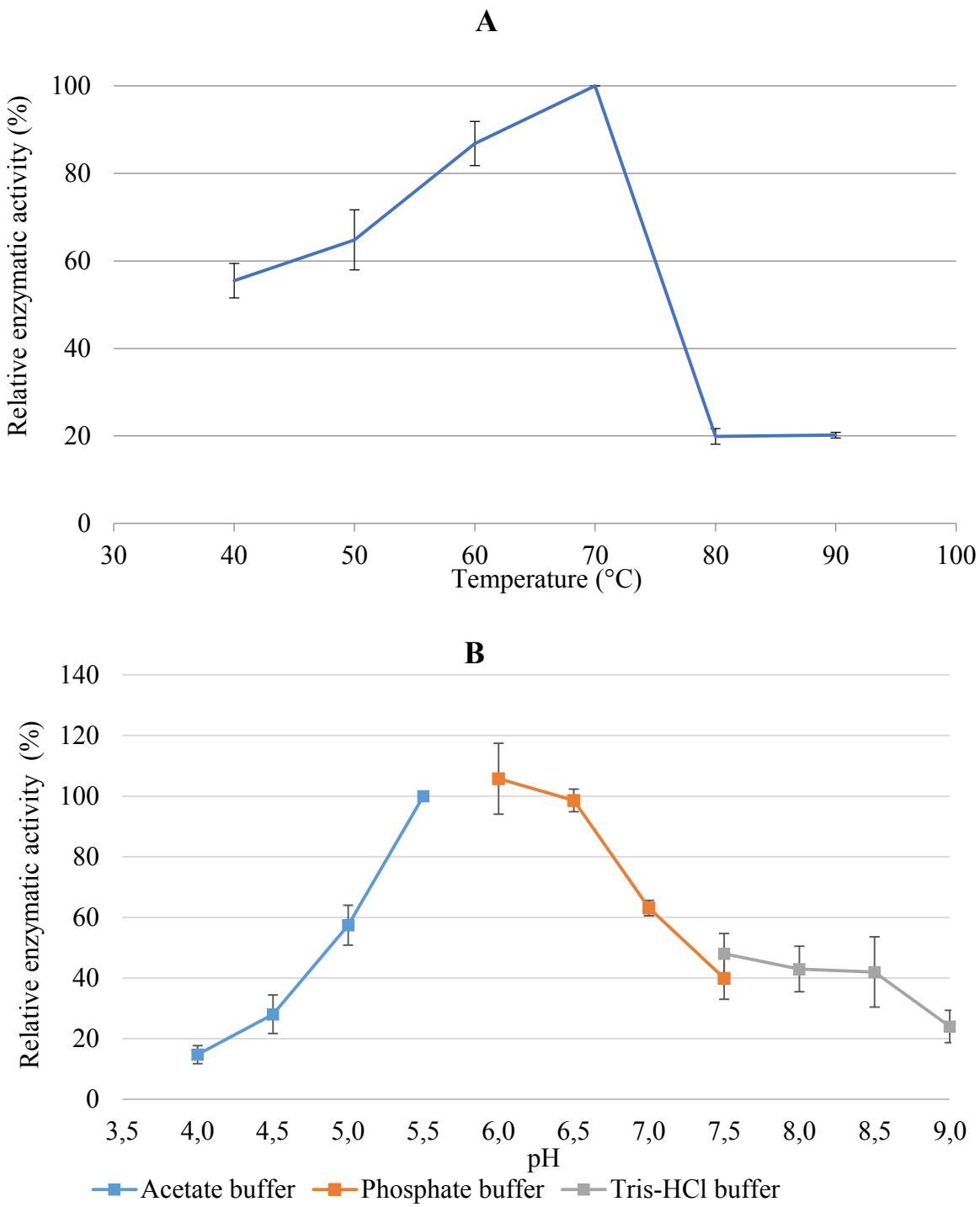

Figure 4.6: Activity profiles of the semi-purified pullulanase of *T. thermophilus* HB8 produced by *P. pastors* Mut^{S}. The pullulanase activity observed at 70°C and pH 5.5, was taken as 100%. Bars represent the standard deviation of the mean of three assays. A: temperature-actvity profile. B: pH-activity profile.

4.4. Conclusions

In this study, thermostable *T. thermophilus HB8* pullulanase type 1 was produced by Mut^s clones of *Pichia pastoris.* The enzyme was found most active in its dimeric form, which most probably explains its intracellular accumulation. By supplying the cultures with TX100 and natural osmolytes, some of the recombinant pullulanase could accumulate extracellularly. Maximal extracellular pullulanase production/accumulation occurred by using 0.4% (w/v) of K-glutamate, and 2% (v/v) of TX100 and amounted to15.47 U/mL of total culture and 72.0% of the enzyme was found in the extracellular fraction. Our results indicated clearly that the methylotrophic yeast *Pichia pastoris* can produce significant amounts of the *T. thermophilus* HB8 type 1 pullulanase but that special means need to be employed in order to release the enzyme into the supernatant fluid. We sugget that the newly developed procedure can be used during industrial, large-scale production of the enzyme for future technical applications.

Acknowledgments

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Chapitre 5. Conclusions et perspectives

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Les pullulanases thermoduriques (ou thermostables) sont des enzymes nécessaires dans de nombreuses applications industrielles. À ce jour, une seule pullulanase, celle de *Bacillus acidopullulyticus*, est disponible sur le marché à des fins industrielles. La présente thèse de doctorat s'inscrivait dans le cadre général du développement industriel d'amylases thermostables, principalement de la pullulanase type 1 de *Thermus thermophilus* HB8.

5.1. Conclusions

Une revue de littérature a été effectuée en cours de ce projet pour identifier les avancées technologiques développées jusqu'à présent pour la production extracellulaire de pullulanases thermostables et les problèmes rencontrés qui empêchent la réalisation de cet objectif.

En effet, il a été constaté que peu de travaux de recherches ont porté sur la possibilité de production directe de pullulanases par leurs hôtes thermophiles d'origine. Nos travaux ont réussi à optimiser la production d'amylases juste par la modification des conditions environnementales. Cependant, ces travaux n'ont pas abordé l'aspect mise à l'échelle des procédés proposés. Cela peut être expliqué par la forte évaporation rencontrée lors de la culture cellulaire, ce qui nécessite l'utilisation d'équipements capables de réduire ce phénomène.

Concernant la production hétérologue des pullulanases thermostables, il a été constaté que plusieurs aspects doivent être considérés, allant de la structure moléculaire de la protéine cible, à l'optimisation des codons selon la souche d'expression, le choix du vecteur d'expression (ex. séquence de sécrétion, promoteur, souche microbienne, gène marqueur), jusqu'aux conditions environnementales. Malgré l'identification de plusieurs vecteurs efficace de production extracellulaire de pullulanases, la structure moléculaire de celle-ci a une influence importante sur la sécrétion de la protéine. En effet, il a été observé que plus la pullulanase est thermostable, plus elle est hydrophobe et peut former des agrégats au niveau intracellulaire, ce qui peut empêcher sa production extracellulaire.

Des méthodes de plan rationnel (*rational design*) et d'évolution dirigée (*directed evolution*) ont été utilisées pour la modification structurale de plusieurs pullulanases pour une meilleure thermostabilité, une meilleure activité catalytique, et/ou une meilleure sécrétion. À travers

les travaux de recherches consultés, il a été observé qu'il est difficile de réaliser un consensus entre les trois aspects : thermostabilité, activité catalytique et sécrétion, pour le développement d'une pullulanase idéale. Il a été constaté qu'il faut développer d'autres outils bioinformatiques capables de prédire de façon efficace l'effet d'une modification structurale sur les aspects précités.

Plusieurs questions furent posées au cours de ce projet de recherche, auxquelles nous avons essayé de répondre.

5.1.1. Est-ce possible de convertir *Thermus thermophilus* HB8 en producteur industriel des amylases, principalement de la pullulanase type 1 ?

Actuellement, plusieurs entreprises aimeraient produire des enzymes de grade technique par la souche sauvage, sans passer par aucune modification génétique, dû au fait que cela présente différents avantages, principalement l'obtention du « label vert », et la production de « cockail » d'enzymes qui pourrait être exploitée dans différentes applications industrielles. Dans ce contexte, notre étude visait d'abord à transformer *Thermus thermophilus* HB8 en producteur industriel, et encourager ainsi d'autres chercheurs pour réaliser des travaux similaires avec d'autres microorganismes thermophiles, surtout qu'à travers la recherche bibliographique effectuée, il y a une perception générale que, malgré le fait que les thermophiles sont une source d'enzymes très intéressantes, ils ne peuvent pas être utilisés comme producteurs de ces enzymes.

Notre travail a démontré que, par l'adoption d'une stratégie d'optimisation, en exploitant des outils statistiques efficaces, tels le plan factoriel et le plan composite central, une grande amélioration de la production de biomolécules par un thermophile peut être réalisée. En effet, il a été démontré que les conditions environnementales sont des paramètres critiques et que, par leur optimisation, le niveau de production de l'enzyme d'intérêt peut être grandement amélioré. Cette étude a permis de confirmer qu'effectivement *T. thermophilus* HB8 est capable de produire une faible activité amylolytique. Le niveau de production de cette dernière a été augmenté plus de 70 fois par une optimisation statistique des conditions environnementales. Le résultat obtenu ne permet pas de conclure sur la capacité de *T. thermophilus* HB8 de produire une pullulanase type 1, dû au fait qu'aucune méthode de concentration et de purification n'a été effectuée lors de la présente étude.

Un procédé de production d'amylase(s) qui peuvent être utilisées dans différents domaines industriels, principalement la formulation de détergents, a été développé dans ce projet.

La culture de *T. thermophilus* HB8, dans un milieu composé de 40 g/L d'extrait de levure, 41 g/L de tryptone, 2.0 g/L de NaCl, 25 g/L d'amidon, et 20% (v/v) de préculture, incubée à 70°C, pendant 5 jours, permet la production extracellulaire d'une activité amylolytique d'un niveau de 76 U/mL, concrètement, d'une activité de conversion de 3 mg d'amidon /min*mL de préparation d'amylases. La comparaison de ce niveau de production avec celui de *Bacillus halodurans* ATCC 21591, une souche alcaline connue comme un « bon producteur » d'amylases, a permis de conclure que le transfert technologique des conditions opératoires optimisées précitées à grande échelle pour la production de(s) amylase(s) par *T. thermophilus* HB8 est prometteur.

La préparation d'amylase(s) produite est caractérisée par une thermostabilité remarquable à une température de 80°C, et par une haute stabilité à différents pH, allant de 4.0 jusqu'à 10.0.

5.1.2. Est-ce que *Pichia pastoris* est capable de produire de manière extracellulaire la pullulanase type 1 de *Thermus thermophilus* HB8 ?

D'après la littérature, la pullulanase type 1 de *T. thermophilus* HB8 est une enzyme caractérisée par des propriétés physico-chimiques très intéressantes, principalement de thermostabilité, la rendant une bonne candidate pour différentes applications, principalement celle de conversion de l'amidon. La séquence primaire de cette enzyme est 100% similaire à celle de *B. thermoleovorans*. Selon la littérature, la production recombinante de cette dernière par *E. coli* a déjà été évaluée. En effet, par l'utilisation d'*E. coli* comme vecteur d'expression, et d'une séquence de sécrétion de l'α-amylase, la production fut extracellulaire avec un niveau d'activité de 0.45 U/mL de surnageant concentré par évaporation et dialyse (Zouari Ayadi *et al.*, 2008). Cependant, le coefficient de concentration n'était pas mentionné dans cette étude.

Vu que *P. pastoris* est connue comme un « bon producteur » de protéines recombinantes au niveau extracellulaire (M. Ahmad *et al.*, 2014; Beaulieu *et al.*, 2005), cette levure méthylotrophe fut exploitée pour la sur-expression du gène de la pullulanase type 1 de *T. thermophilus*, en exploitant pour la première fois le vecteur pD912. L'effet de six séquences de sécrétion sur la production extracellulaire de la pullulanase recombinante par *P. pastoris* a été évalué. Une faible production extracellulaire de cette enzyme a été observée dans le cas de l'utilisation de la séquence de sécrétion de l'alpha-facteur de *S. cereviciae* (0.14 U/mL du surnageant du milieu de culture sans aucune concentration), malgré que ce peptide signal

a été utilisé grandement dans différentes études, démontrant sa capacité de facilité la sécrétion de la protéine cible.

D'après la recherche bibliographique, les vecteurs d'expression les plus exploités pour la production recombinante par *P. pastoris* sont pPIC9, pPIC9K, et pGAPZalpha (Nisha and Satyanarayana, 2017). Tous ces vecteurs possèdent la séquence de sécrétion de l'alpha-facteur de *S. cerevisiae*. Pour comparer l'effet du vecteur pD912 versus pPIC9K et pGAPZalpha sur la production extracellulaire de la pullulanase type 1 de *T. thermophilus* HB8, au cours de ce projet de thèse, des clones de *P. pastoris* portant pPIC9K-*Pul*, et pGAPZalpha-*Pul* ont été obtenus. Tous les clones sélectionnés produisaient la pullulanase seulement au niveau intracellulaire.

La présente étude a permis la détermination des propriétés physico-chimiques de la pullulanase recombinante produite par *P. pastoris*. Elle a démontré que la forme la plus active de cette enzyme est sa forme dimère (~ 160 kDa). Aussi le fait que cette enzyme soit hautement thermostable (50% de l'activité résiduelle après incubation de l'enzyme à 70°C pendant une durée de 120 min), la rend fort intéressante. Par conséquent, la structure moléculaire de cette pullulanase peut être un paramètre majeur qui empêche la production extracellulaire de celle-ci.

Ce projet de recherche a permis l'identification de plusieurs facteurs qui, malgré la production intracellulaire de la protéine par *P. pastoris,* peuvent être exploités pour améliorer l'accumulation de celle-ci. Pour la première fois une évaluation de l'effet du Triton X-100 sur l'accumulation d'une pullulanase par *P. pastoris* a été étudiée. Le niveau de concentration et le temps d'ajout du Triton X-100 furent évalués. Aussi, les effets d'autres paramètres (des osmolytes naturels, le pH, la température et la source de carbone) ont été évalués. Cette étude présente une nouvelle méthode d'extraction chimique de la pullulanase recombinante en cours du procédé de fermentation avec *P. pastoris*, qui peut être utilisée pour la récupération facile d'autres protéines produites au niveau intracellulaire. Une activité extracellulaire de pullulanase de 15.47 U/mL a pu être obtenue par une culture de *P. pastoris* (80 g de matière sèche/L) après un traitement chimique en parallèle avec la phase d'induction de l'expression du gène pullulanase. Ce traitement est composé de deux phases : la première est l'ajout de l'acide glutamique de potassium à une concentration finale de 0.40% (w/v) avant 5 h d'induction au méthanol, et la deuxième phase est l'ajout de Triton X-100, après

48 h d'induction, à une concentration finale de 2% (v/v) pH 8.0, suivit d'une incubation de 36 h à 30°C, sous une agitation de 1000 rpm.

5.2. Perspectives

Cette thèse avait comme principal objectif la génération d'une preuve de concept visant la production extracellulaire d'une pullulanase thermodurique de *T. thermophilus* HB8 par un procédé de fermentation pouvant être exploité à l'échelle industrielle. Cette étude ouvre un certain nombre d'interrogations et d'autres avenues intéressantes de recherche qui peuvent améliorer les procédés présentés dans cette thèse.

5.2.1. Caractérisation de la structure moléculaire des amylases de *T. thermophilus* HB8

La présente étude a mis en évidence la capacité de *T. thermophilus* HB8 de produire un « cocktail » d'amylase(s) hautement thermostable(s) (100% d'activité relative après 24 h d'incubation à 80°C). Les structures moléculaires de ces enzymes seront très intéressantes à étudier par la suite en vue de comprendre davantage leurs mécanismes réactionnels, et aussi pour le développement d'autres enzymes thermostables. En effet, il existe actuellement différentes techniques de séparation et de concentration (ex. chromatographie, dialyse, microfiltration), qui peuvent être exploitées pour la purification de ces amylases. Aussi, des méthodes d'identification de protéines telles Western blot, zymographie, et protéomique peuvent être utilisées pour l'étude de chacune des amylases produites. L'identification des produits générés après réaction entre ces amylases et des substrats tels l'amidon, et d'autres polysaccharides, permettra la détermination du type d'amylase produite par *T. thermophilus* HB8. La cristallographie aussi une technique efficace qui peut être utilisée pour l'identification de la structure 3D de ces protéines thermostables.et de leurs sites actifs

5.2.2. Production des amylases par *T. thermophilus* HB8 : De la fiole au bioréacteur

L'optimisation du milieu de culture, réalisée au cours de cette étude pour la production d'amylases par *T. thermophilus* HB8 a évaluée indirectement l'effet du ratio carbone/azote sur l'activité amylolytique produite. En effet, les paramètres qui ont fait l'objet d'optimisation, extrait de levure, tryptone et amidon sont des ingrédients complexes sources de carbone et d'azote pour la culture cellulaire. Une caractérisation de ces matières premières par la quantification des différents éléments, principalement la concentration de carbone, d'azote, de phosphore, de potassium, de sodium, de magnésium, de calcium, du fer, et des vitamines, permettra le développement et l'optimisation d'un milieu synthétique qui pourra

être utilisé à l'échelle industrielle. La source d'azote organique ou inorganique peut aussi influencer la production d'amylases. Il y a des études qui ont reporté que chaque microorganisme a plus d'affinité avec un inducteur de production d'amylases donné par rapport à d'autres. Une évaluation de l'effet du type d'inducteur (ex. type d'amidon, type de polysaccharide) pourrait être conduite pour une meilleure production d'amylases.

Aussi, le bioprocédé consolidé (*consolidated bioprocessing*) est une technologie de pointe développée ces derniers années (Behera and Ray, 2016), et qui serait intéressante à exploiter pour l'hydrolyse de l'amidon et la production d'amylases par *T. thermophilus* HB8 en une seule étape.

5.2.3. Influence de la structure moléculaire de la pullulanase type 1 de *T. thermophilus* HB8 sur son niveau de sécrétion

Malgré le fait que la levure *P. pastoris* est connue par sa capacité de production extracellulaire de protéines dont le poids moléculaire peut atteindre 200 kDa, la pullulanase type 1 de *T. thermophilus* HB8 (monomère 80 kDa) fut principalement produite au niveau intracellulaire par cette levure. Le fait que cette enzyme est plus active sous sa forme dimère (160 kDa) peut expliquer ce résultat. Il serait intéressant d'élucider mieux la structure moléculaire de la pullulanase type 1 de *T. thermophilus* HB8 pour mieux comprendre son mécanisme réactionnel et aussi déterminer si sa structure réduit sa sécrétion au niveau extracellulaire.

Pechan *et al.,* (2004) ont observé que le domaine N-terminal de la pullulanase peut empêcher sa sécrétion même si celle-ci est bien fusionnée avec un SP connu par son pouvoir sécrétoire. Par la modification ou l'élimination du domaine N-terminal pourrait-on améliorer la sécrétion de la pullulanase type 1 de *T. thermophilus* HB8 par *P. pastoris* ? De plus, les techniques de *protein engineering* peuvent être exploitées pour réduire son poids moléculaire pour améliorer sa sécrétion et augmenter son activité catalytique, et sa thermostabilité.

5.2.3. Triton X-100 : extracteur de protéine

Lors de l'étude de l'effet du Triton X-100 sur l'extraction de la pullulanase de *P. pastoris*, il a été observé que, malgré l'augmentation de la quantité de biomasse d'environ 2 fois, l'activité enzymatique n'a pas augmenté. L'évaluation de l'effet des ratios Triton X-100/biomasse et Triton X-100/pullulanase est importante pour avoir une meilleure accumulation extracellulaire de la pullulanase.

Selon la littérature, le Triton X-100 peut colmater les membranes de dialyses et de microfiltration. Il faut donc développer des méthodes chimiques ou physiques qui peuvent éliminer le Triton X-100 avant de passer aux étapes de récupération et de concentration de la protéine cible.

Pour réduire le temps de fermentation de *P. pastoris* et améliorer la production de la pullulanase, une expression constitutive pourrait être exploitée. En effet, Nisha and Satyanarayana (2017) ont observé que l'expression constitutive du gène de l'amylopullulanase de *Geobacillus thermoleovorans* était supérieure par rapport à celle inductive. Cela réduira le temps de production de cinq (05) jours de fermentation, dans le cas d'une production inductive, à une de trois (03) jours dans le cas d'une production constitutive.

Annexe

Annexe : Étapes de clonage du gène pullulanase (travail effectué par CNETE, Shawinigan)

Sommaire des étapes

1- Synthèse chimique du gène de la pullulanase par la compagnie DNA 2.0
2- Arrivée du gène dans un plasmide-mère, le pM269
3- Transformation (pM269 dans *E. coli* DH5a)
4- Stocks Glycérol et Miniprep des clones pM269 / *E. coli* DH5a
5- Vérification des minipreps des clones par dosage d'ADN et par séparation sur gel d'agarose
6- Transfert du gène de pM269 dans 10 pDAUGHTER
7- Transformation des pDAUGHTER+gène dans *E. coli* DH5a
8- Stocks Glycérol et Miniprep des clones pDAUGHTER + gène / *E. coli* DH5a
9- Préparer des cellules de *Pichia pastoris* mutS électrocompétentes
10- Linéarisation de chaque pDAUGHTER gène avec Sac1 et transformation dans *P. pastoris*
11- Sélection des clones sur antibiotique déterminé (zéocine) et réalisation des tests enzymatiques

Étape 1 – Synthèse du gène

- Synthèse de la séquence du gène pullulanase chez DNA 2.0. Le coût est de 0,65$ par paire de bases.

Étape 2 – Réhydratation du pM269 + gène

- Arrivée du gène dans un plasmide-mère (pM269). Plasmide imbibé et séché sur un papier buvard circulaire de 15 mm de diamètre et stérile. En principe, le papier buvard contient entre 2-5 µg de plasmide au total.

- Découpage du papier en languettes avec un scalpel stérile, puis son dépôt dans un tube Eppendorf de 1,5 mL, stérile.

- Réhydratation de l'ADN avec 100 µL de Tris-HCl, 10 mM, pH 7,6, stérile, pendant une durée de 5 minutes.

- Transfert du papier imbibé dans une colonne de 1,5 mL. Cette colonne servira comme grillage pour bloquer le papier et permettra l'élution du 100 µL + ADN ci-haut. Centrifuger, 1 min @15 000g.

- Dosage de l'ADN élué à 260 nm et son stockage à -20°C.

Étape 3- Transformation du pM269-Pul/ DH5a

1. Décongélation des cellules et leur maintien froid dans la glace pendant 10 min.

2. Ajout de 1-5 µL de l'ADN plasmidique aux cellules d'*E. coli* DH5a (environ 1 pg – 100 ng ADN).

3. Maintien du mélange sur la glace pendant 30 minutes.

4. Application d'un choc thermique à exactement 42°C pour exactement 30 secondes.

5. Maintien du mélange sur la glace pour 5 minutes, sans mélanger.
6. Ajout de 950 µL de milieu SOC (température pièce) aux cellules.
7. Incubation à 37°C pendant 60 minutes en agitant à 250 rpm.
8. Application des dilutions au besoin sur le milieu SOC.
9. Inoculation de 100 µL sur géloses LB sélectives (33 ug/l de chloramphénicol) et incubation à 37°C pendant 18h.
10. Décompte des colonies transformées / DH5a.

Recette Milieu SOC

- Tryptone 2,0 %
- Extrait de levure 0,5 %
- NaCl 0,058 %
- KCl 0,018 %
- Autoclaver, 15 min @ 121°C, cycle liquide
- $MgCl_2$ 0,094 %
- Glucose 0,360 %

Étape 4- Stock glycérol et Miniprep des clones

- Repiquage des clones isolés sur LB et incubation à 37°C pendant 24h.

- Lancement d'une culture des colonies dans 5 ml de LB dans un tube Falcon stérile de 50 mL, et incubation à 30°C pendant 16h.

- Centrifugation des tubes à 5000*g pendant 5 min et récupération du culot.

- Extraction des plasmides avec un kit *Plasmid Mini Kit I* de la compagnie Omega Biotek. Elution se réalise dans 70 µL EB.

Étape 5- Digestion Xba1 des minipreps + gel agarose

-Dosage de l'ADN.

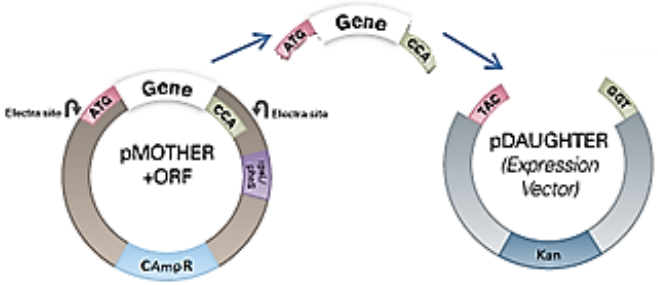
- Séparation sur gel d'agarose : une digestion de 0.2 µL de plasmide avec l'enzyme Xba1 à 37°C pendant 20 min. Ajout aussi d'un marqueur 2-log. La migration est réalisée à 120V pendant 50 minutes. Ensuite, dépôt sur gel agarose 0,8 %.

Recette : 2,0 µL ADN (**~700 à 900 ng)**
1,0 µL Tampon Cutsmart 10X
2,0 µL Enzyme Xba1 (NEB # R0145S) (40 U)
5,0 µL H2O distillée DEPC
10 µL Total

Étape 6- Transfert du gène de pM269 à pDAUGHTER (10x)

- Utilisation du kit IP-Free© Electra DAUGHTER™ Vectors– *Pichia* de la compagnie DNA 2.0. Ce kit contient une enzyme de restriction de type IIS (Electra Enzyme SapI). Incubation du mélange à la température de la pièce pendant 20 min.

Solution	Volume (ul)
Plasmide pM269 (20 ng d'ADN)	1
Plasmide-fille (20ng d'ADN)	1
Tampon Electra	2
Enzyme de restriction Electra	1
H_2O stérile	15



Étape 7- Transformation

- Transformation de 2 µL de chaque ligation selon les étapes 3 et 4.

Le milieu de gélose sélectif est LB-low salt* avec 50 µg/mL de l'antibiotique zéocine.

Recette LB standard :	Tryptone	10 g/L
	Extrait de levure	5 g/L
	NaCl	10 g/L
	Agar	15 g/L

* LB *low salt* est le même milieu sauf 5 g/L de NaCl au lieu de 10 g/l, car cela affecte l'efficacité de la zéocine.

- Décompte des colonies transformées / DH5a.

Étape 8 Stocks Glycérol et Miniprep des clones pDAUGHTER + gène / *E. coli* DH5a

- Repiquage des clones pour chacune des transformations des pDAUGHTER + gène / DH5a sur géloses de LB low salt Zeo 50. Et incubation à 37°C pendant 24h.

- Pour chaque clone, lancement de deux culture de LB-low salt, une de 5 ml pour l'extraction du plasmide, et l'autre de 10 ml pour la préparation de stocks en glycérol. Une incubation est appliquée aux cultures à 37°C, avec une agitation de 200 rpm, pendant 18h.

- Pour les stocks des clones transformés dans le glycérol, une centrifugation sera appliquée aux différentes cultures à 5000*g pendant 5 min. Les culots obtenus seront suspendus dans 1 ml d'une solution de 30% (v/v) de glycérol. Ensuite, distribution d'aliquotes de 500µl dans des tubes cryogéniques.

- Stockage à -80°C.

- Extraction des plasmides par le kit de minipréps mini kit 1 d'Omega Biotek (D6942-01).

- Dosage de l'ADN à 260 nm.

Étape 9 - Transformation de *Pichia pastoris MutS* avec pDAUGHTER

Préparation des solutions et milieux de culture

1. ***YPD Broth* (*pour préculture et culture de Pichia)***

   - Extrait de levure 1 % ou 10 g/L
   - Bacto-Peptone 2 % ou 20 g/L
   - Glucose 2 % ou 20 g/L
   - Eau distillée pour compléter au volume

2. ***BEDS solution* (*pour lavage des cellules)***

   - Bicine-NaOH pH 8,3 10 mM ou 1,63 g/L ajusté à pH 8,3 avec NaOH
   - Ethylene Glycol 3 % ou 30 mL/L
   - DMSO 5 % ou 50 mL/L
   - Sorbitol 1 M ou 182,17 g/L
   - Eau distillée pour compléter au volume

3. ***DTT 1M* (*Dithiothréitol, agent réducteur fort, antioxydant)***
   Préparer 10 mL. Masse molaire = 154,25 g/mol, donc peser 1,54 g dans 10 mL et filtrer 0,22 µM
4. ***YPDS agar + Zéocine* (*Géloses sélectives pour étaler nos transfos)***
   - Extrait de levure 1 % ou 10 g/L
   - Bacto- Peptone 2 % ou 20 g/L
   - Sorbitol 1 M ou 182,17 g/L
   - Agar 1,5 % ou 15 g/L
   - Glucose 2 % ou 20 g/L
   - Eau distillée pour compléter au volume
5. ***Solution mix* (*YPD 50% + Sorbitol 1M 50%)* (*Pour levures après électroporation)***
   Mixer 50 % v/v de YPD, préparé au point 1 + 50% de Sorbitol 1M. Filtrer 0,22 µM

Préparation de cellules électrocompétentes

1. ***Repiquage sur gélose fraîche***

- 2 jours avant (48h), repiquage de la souche de levure (*Pichia pastoris Mut S, #DNA 2.0 PPS-9011)* sur une gélose YPD, à partir du stock glycérol -80°C et incubation à 30°C pour 36 à 48h.

2. ***Démarrage de la pré-culture***

- À partir de la culture de *P. pastoris* sur gélose YPD (36h de croissance), lancement de la pré-culture effectué en inoculant une colonie dans 10 mL de bouillon YPD en flacon 50 mL. Ensuite, application d'une incubation à 30 °C, 200 RPM pendant 18h.

3. ***Démarrage de la culture***

- Mesure de la DO600 de la pré-culture, qui doit être environ 14 :
- Selon le protocole DNA 2.0, la culture doit être démarrée à 0,2 UDO. Dilution de la pré-culture ;
- Démarrage de la culture à 30°C, 220 RPM et suivi de la DO600 jusqu'à l'atteinte de 0,8-1,0 ;
- À l'atteinte de la DO, transfert de la culture dans 4 tubes de 50 mL, puis centrifugation 5 min à 500g.
- Suspension du culot dans 9 mL de solution BEDS et dans 1 mL de DTT 1 M.
- Incubation 5 min à 37 °C sous agitation (100 RPM).
- Centrifugation 5 min. à 500 x g.
- Re-suspension du culot dans 4 mL de solution BEDS, sans ajout de DTT.
  À ce point, les cellules sont compétentes.
- Prise d'aliquotes de 40 µL de cellules dans des tubes Eppendorf stériles. 20 aliquotes de 40 µL sont conservés sur glace pour faire les transformations.

Transformation des plasmides dans *Pichia*

1- ***Électroporation des cellules avec les plasmides***

- Mise des tubes de cellules sur glace
- Pour chaque transformation de 40µL de cellules compétentes, ajout de 1,5 µL (50-100ng) du plasmide linéaire. La linéarisation est effectuée par un kit de Digestion SacI.
- Tranfert du contenu des tubes dans des cuvettes à électroporation froides (2 mm Biorad Cat #165-2086).
- Application d'une électroporation sur le Gene Pulser II, Bio-Rad : 1500 V; 200 Ω; 25 µF; cuvettes de 2 mm.
- Immédiatement, un ajout de 1 mL d'une solution de 50% YPD, 50% Sorbitol 1M est effectué.
- Transfert du contenu des cuvettes dans des tubes Falcon stériles de 15 mL, puis incubation pendant 1h à 30 °C sous une agitation de 300 RPM.
- Ensemencement de 100 µL de chaque transformation sur géloses YPDS contenant 100, 200 et 500 µg/mL de zéocine (Invitrogen, via Life technologies #R25001).
- Incubation des géloses à 30 °C pendant 2-3 jours.
- Décompte des colonies et repiquage des clones

Étape 10 – Sélection des clones transformés et réalisation des tests enzymatiquese

- Repiquage de chaque clone transformé sur gélose dans 5 ml de bouillon BMGY dans un tube de 50 ml. Incubation à 30 °C sous agitation de 200 RPM, jusqu'à avoir une DO_{600} de 2-6 (16-18 heures).
- Centrifugation à 3000 RPM, 5 min, puis suspension du culot dans 5 ml de bouillon BMMY.

- Incubation à 30 °C sous agitation (200 RPM) et ajout direct du méthanol 100 % à une concentration finale de 0,5 % à chaque 24 h afin de maintenir l'induction. Un prélèvement de 1 ml est effectué à 0, 24, 48, 72, 96, 120 et 144 h pour la réalisation des différents tests.

Tableau 0.1: Composition du milieu de croissance cellulaire BMGY et du milieu d'induction BMMY

BMGY (pour pré-culture, milieu complexe)	**BMMY** (pour induction, milieu complexe)
1 % Extrait de levure	1 % Extrait de levure
2 % peptone	2 % peptone
100 mM potassium phosphate, pH 6,0	100 mM potassium phosphate, pH 6,0
13,4 g/L *Yeast Nitrogen Base* sans acides aminés	13,4 g/L *Yeast Nitrogen Base* sans acides aminés
0,4 mg/L biotine	0,4 mg/L biotine
1 % glycérol	0,5 % méthanol

Liste des références

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Acer Ö, Bekler FM., Pirinççioğlu H., et al., (2016) Purification and characterization of thermostable and detergent-stable α- amylase from Anoxybacillus sp . AH1. Food Technol Biotechnol 54:70–77. doi: 10.17113/ftb.54.01.16.4122

Ahmad, M., Hirz, M., Pichler, H., Schwab, H., (2014). Protein expression in Pichia pastoris: recent achievements and perspectives for heterologous protein production. Appl. Microbiol. Biotechnol. 98, 5301–5317. https://doi.org/10.1007/s00253-014-5732-5.

Ahmad, N., Rashid, N., Haider, MS., Akram, M., Akhtar, M., (2014). Novel Maltotriose-Hydrolyzing Thermoacidophilic Type III Pullulan Hydrolase from Thermococcus kodakarensis. Appl. Environ. Microbiol. 80, 1108–1115. https://doi.org/10.1128/AEM.03139-13.

Akeboshi, H., Kashiwagi, Y., Aoki, H., Tonozuka, T., Nishikawa, A., Sakano, Y., (2003). Construction of an efficient expression system for Aspergillus isopullulanase in Pichia Pastoris, and simple purification methode. Biosci. Biotechnol. Biochem. 67, 1149–1153.

Albertson, G.D., McHale, R.H., Gibbs, M.D., Bergquist, P.L., (1997). Cloning and sequence of a type I pullulanase from an extremely thermophilic anaerobic bacterium, Caldicellulosiruptor saccharolyticus. Biochim. Biophys. Acta - Gene Struct. Expr. 1354, 35–39. doi:10.1016/S0167-4781(97)00123-1

Amon, A., Berk, A., Bretscher, A., Kaiser, C., Krieger, M., Lodish, H., Ploegh, H., Scott, M., (2004). Biologie moléculaire de la cellule.4th ed, De Boeck.

Antranikian, G., Zablowski, P., Gottschalk, G., (1987). Conditions for the overproduction and excretion of thermostable α-amylase and pullulanase from Clostridium thermohydrosulfuricum DSM 567. Appl. Microbiol. Biotechnol. 27, 75–81. doi:10.1007/BF00257257

Arikan B (2008) Highly thermostable, thermophilic, alkaline, SDS and chelator resistant amylase from a thermophilic Bacillus sp. isolate A3-15. Bioresour Technol 99:3071–3076. doi: 10.1016/j.biortech.2007.06.019

Beaulieu, L., Groleau, D., Miguez, C.B., Jetté, J.-F., Aomari, H., Subirade, M., (2005). Production of pediocin PA-1 in the methylotrophic yeast Pichia pastoris reveals unexpected inhibition of its biological activity due to the presence of collagen-like material. Protein Expr. Purif. 43, 111–125. doi:10.1016/j.pep.2005.05.012

Ben Messaoud, E., Ben Ammar, Y., Mellouli, L., Bejar, S., (2002). Thermostable pullulanase type I from new isolated Bacillus thermoleovorans US105: cloning, sequencing and expression of the gene in E. coli. Enzyme Microb. Technol. 31, 827–832. https://doi.org/10.1016/S0141-0229(02)00185-0.

Berger JL, Lee BH, Lacroix C., (1995) Identification of new enzyme activities of several strains of Thermus species. Appl Microbiol Biotechnol 44:81–87. doi: 10.1007/BF00164484

Bertoldo, C., Armbrecht, M., Becker, F., Schäfer, T., Antranikian, G., Liebl, W., (2004). Cloning, Sequencing, and Characterization of a Heat- and Alkali-Stable Type I Pullulanase from Anaerobranca gottschalkii. Appl. Environ. Microbiol. 70, 3407–3416. doi:10.1128/AEM.70.6.3407-3416.2004

Bertoldo, C., Duffner, F., Jorgensen, P.L., Antranikian, G., (1999). Pullulanase Type I from Fervidobacterium pennavorans Ven5: Cloning, Sequencing, and Expression of the Gene and Biochemical Characterization of the Recombinant Enzyme. Appl. Environ. Microbiol. 65, 2084–2091.

Bommarius, A.S., Blum, J.K., Abrahamson, M.J., (2011). Status of protein engineering for biocatalysts: how to design an industrially useful biocatalyst. Curr. Opin. Chem. Biol. 15, 194–200. doi:10.1016/j.cbpa.2010.11.011

Brocca, S., Schmidt-Dannert, C., Lotti, M., Alberghina, L., Scmid, R., (1998). Design, total synthesis, and functional overexpression of the Candida rugosa lip1 gene coding for a major industrial lipase. Protein Sci 7, 1415–1422.

Montgomery CD (2012) Design and Analysis of Experiments, 8th edn. Wiley, New York

Cava F, Hidalgo A, Berenguer J (2009) Thermus thermophilus as biological model. Extremophiles 13:213–231. doi: 10.1007/s00792-009-0226-6

Chen, A., Li, Y., Liu, X., Long, Q., Yang, Y., Bai, Z., (2014). Soluble expression of pullulanase from Bacillus acidopullulyticus in Escherichia coli by tightly controlling basal expression. J. Ind. Microbiol. Biotechnol. 41, 1803–1810. https://doi.org/10.1007/s10295-014-1523-3.

Chen, A., Li, Y., Nie, J., McNeil, B., Jeffrey, L., Yang, Y., Bai, Z., (2015). Protein engineering of Bacillus acidopullulyticus pullulanase for enhanced thermostability using in silico data driven rational design methods. Enzyme Microb. Technol. 78, 74–83. https://doi.org/10.1016/j.enzmictec.2015.06.013.

Chen, A., Sun, Y., Zhang, W., Peng, F., Zhan, C., Liu, M., Yang, Y., (2016). Downsizing a pullulanase to a small molecule with improved soluble expression and secretion efficiency in Escherichia coli. Microb. Cell Fact. 15, 1–10. doi:10.1186/s12934-015-0403-5

Chen, W.-B., Nie, Y., Xu, Y., (2013). Signal peptide-independent secretory expression and characterization of pullulanase from a newly isolated Klebsiella variicola SHN-1 in Escherichia coli. Appl. Biochem. Biotechnol. 169, 41–54. doi:10.1007/s12010-012-9948-5

Chen, X., Hu, Y.H., Chen, W. Di, Li, W. Di, Huang, Z.C., Li, Y., Luo, Y.W., Huang, Y.X., Chen, Y.T., Wang, K., Li, L., (2016). Comparison of inducible versus constitutive expression of plectasin on yields and antimicrobial activities in Pichia pastoris. Protein Expr. Purif. 118, 70–76. doi:10.1016/j.pep.2015.10.010

Choi, K.H., Cha, J., (2015). Membrane-bound amylopullulanase is essential for starch metabolism of Sulfolobus acidocaldarius DSM639. Extremophiles 19, 909–920. doi:10.1007/s00792-015-0766-x

Cori, G.., Larner, J., (1951). Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. Biol. Chem. 188, 17.

Crolla A, Kennedy KJ (2001) Optimization of citric acid production from Candida lipolytica Y-1095 using n-paraffin. J Biotechnol 89:27–40. doi: 10.1016/S0168-1656(01)00278-4

Delic, M., Valli, M., Graf, A.B., Pfeffer, M., Mattanovich, D., Gasser, B., (2013). The secretory pathway: exploring yeast diversity. FEMS Microbiol. Rev. 37, 872. doi:10.1111/1574-6976.12020

Deng Z., Yang, H., Shin, HD., Li, J., Liu, L., (2014). Structure-based rational design and introduction of arginines on the surface of an alkaline alpha-amylase from Alkalimonas amylolytica for improved thermostability. Appl. Microbiol. Biotechnol. 98(21):8937-45. doi: 10.1007/s00253-014-5790-8.

De Souza PM., de Oliveira Magalhães P., (2010). Application of microbial α-amylase in industry A review. Brazilian Journal of Microbiology. 41(4):850-861. doi:10.1590/S1517-83822010000400004.

Diamant, S., Rosenthal, D., Azem, A., Eliahu, N., Ben-Zvi, A.P., Goloubinoff, P., (2003). Dicarboxylic amino acids and glycine-betaine regulate chaperone-mediated protein-disaggregation under stress. Mol. Microbiol. 49, 401–410.

Domań-Pytka, M., Bardowski, J., (2004). Pullulan Degrading Enzymes of Bacterial Origin. Crit. Rev. Microbiol. 30, 107–121. https://doi.org/10.1080/10408410490435115.

Duan, X., Chen, J., Wu, J., (2013a). Improving the thermostability and catalytic efficiency of Bacillus deramificans pullulanase by site-directed mutagenesis. Appl. Environ. Microbiol. 79, 4072–4077. https://doi.org/10.1128/AEM.00457-13

Duan, X., Chen, J., Wu, J., (2013b). Optimization of pullulanase production in Escherichia coli by regulation of process conditions and supplement with natural osmolytes. Bioresour. Technol. 146, 379–385. https://doi.org/10.1016/j.biortech.2013.07.074.

Duan, X., Zou, C., Wu, J., (2015). Triton X-100 enhances the solubility and secretion ratio of aggregation-prone pullulanase produced in Escherichia coli. Bioresour. Technol. 194, 137–143.

Duffner, F., Bertoldo, C., Andersen, J.T., Wagner, K., Antranikian, G., (2000). A New Thermoactive Pullulanase from Desulfurococcus mucosus: Cloning, Sequencing, Purification, and Characterization of the Recombinant Enzyme after Expression in Bacillus subtilis. J. Bacteriol. 182, 6331–6338. doi:10.1128/JB.182.22.6331-6338.2000

Elleuche, S., Schröder, C., Sahm, K., Antranikian, G., (2014). Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. Curr. Opin. Biotechnol. 29, 116–123. doi:10.1016/j.copbio.2014.04.003

Farhat-Khemakhem, A., Ali, M.B., Boukhris, I., Khemakhem, B., Maguin, E., Bejar, S., Chouayekh, H., (2013). Crucial role of Pro 257 in the thermostability of Bacillus phytases: biochemical and structural investigation. Int. J. Biol. Macromol. 54, 9–15.

Farris, PL., (2009). Starch: Economic Growth and organisation of the US corn Starch industry, Chapitre 2. BeMiller, J.;Whistler, R. Starch chemistry and technology.

Fatoni, A., Zusfahair, (2012). Thermophilic amylase from Thermus sp. isolation and its potential application for bioethanol production. Songklanakarin J. Sci. Technol. 34, 525–531.

Farhat-Khemakhem, A., Ali, MB., Boukhris, I., Khemakhem, B., Maguin, E., Bejar, S., Chouayekh, H., (2013). Crucial role of Pro 257 in the thermostability of Bacillus phytases: biochemical and structural investigation. Int. J. Biol. Macromol. 54, 9–15.

Fujiwara, S., (2002). Extremophiles. J. Biosci. Bioeng. 94, 518.

Gantelet, H., Duchiron, F., (1998). Purification and properties of a thermoactive and thermostable pullulanase from Thermococcus hydrothermalis, a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent. Appl. Microbiol. Biotechnol. 49,

770–777. https://doi.org/10.1007/s002530051245.

Gomes, I., Gomes, J., Steiner, W., (2003). Highly thermostable amylase and pullulanase of the extreme thermophilic eubacterium Rhodothermus marinus: production and partial characterization. Bioresour. Technol. 90, 207–214. doi:10.1016/S0960-8524(03)00110-X

Gupta, G.N., Srivastava, S., Khare, S.K., Prakash, V., (2014). Extremophiles: An Overview of Microorganism from Extreme Environment. Int. J. Agric. Environ. Biotechnol. 7, 371.

Han, T., Zeng, F., Li, Z., Liu, L., Wei, M., Guan, Q., Liang, X., Peng, Z., Liu, M., Qin, J., Zhang, S., Jia, B., (2013). Biochemical characterization of a recombinant pullulanase from Thermococcus kodakarensis KOD1. Lett. Appl. Microbiol. 57, 336–343. doi:10.1111/lam.12118

Heinzelman, P., Snow, C.D., Wu, I., Nguyen, C., Villalobos, A., Govindarajan, S., Minshull, J., Arnold, F.H., (2009). A family of thermostable fungal cellulases created by structure-guided recombination. Proc. Natl. Acad. Sci. U. S. A. 106, 5610–5615. doi:10.1073/pnas.0901417106

Helenius, A., Simons, K., (1975). Solubilization of membranes by detergents. BBA - Rev. Biomembr. 415, 29–79. https://doi.org/10.1016/0304-4157(75)90016-7.

Hii, LS., Rosfarizan, M., Ling, TC., Ariff, AB., (2012a). Statistical Optimization of Pullulanase Production by Raoultella planticola DSMZ 4617 Using Sago Starch as Carbon and Peptone as Nitrogen Sources. Food Bioprocess Technol. 5, 729–737. doi:10.1007/s11947-010-0368-7

Hii, LS., Tan, JS., Ling, TC., Ariff, AB., (2012b). Pullulanase: role in starch hydrolysis and potential industrial applications. Enzyme Res. 2012, 921362. doi:10.1155/2012/921362

Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D., (2004). Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris. Biotechnol Bioeng 85, 367–375.

Hiromi, K., Onishi, M., Kanaya, K., Matsumoto, T., (1975). The pH Jump Study of Enzyme Proteins I . Liquefying a-Amylase from Bacillus subtilis. Biochem J 77, 957–963.

Ibrahim, M.H.A., Steinbüchel, A., (2010). High-Cell-Density Cyclic Fed-Batch Fermentation of a Poly(3-Hydroxybutyrate)-Accumulating Thermophile, Chelatococcus sp. Strain MW10. Appl. Environ. Microbiol. 76, 7890–7895. https://doi.org/10.1128/AEM.01488-10

Janse, BJ., Pretorius, IS., (1993). Expression of the Klebsiella pneumoniae pullulanase-encoding gene in Saccharomyces cerevisiae. Curr.Genet. 24, 32–37. doi:10.1007/BF00324662

Jasilionis, A., Petkauskaite, R., Kuisiene, N., (2014). A novel type I thermostable pullulanase isolated from a thermophilic starch enrichment culture. Microbiology 83, 227–234. https://doi.org/10.1134/S0026261714030084

Kim, JH., Kim, HR., Lim, MH., Ko, HM., Chin, JE., Lee, HB., Kim, IC., Bai, S., (2010). Construction of a direct starch-fermenting industrial strain of Saccharomyces cerevisiae producing glucoamylase, alpha-amylase and debranching enzyme. Biotechnol. Lett. 32, 713–719. doi:10.1007/s10529-010-0212-1

Kolawole, AO., Ajele, JO., Sirdeshmukh, R., (2011). Purification and characterization of alkaline-stable β-amylase in malted African finger millet (Eleusine coracana) seed. Process Biochem. 46, 2178–2186. https://doi.org/10.1016/j.procbio.2011.08.021

Koley, D., Bard, A.J., (2010). Triton X-100 concentration effects on membrane permeability of a single HeLa cell by scanning electrochemical microscopy (SECM). Proc. Natl. Acad. Sci. U. S. A. 107, 16783–7. https://doi.org/10.1073/pnas.1011614107.

Krahe, M., Antranikian, G., Markl, H., (1996). fermentation of extremophilic microorganisms. Fems Microbiol. Rev. 18, 271–285.

Kumar, L., Awasthi, G., Singh, B., (2011). Extremophiles: a novel source of industrially important enzymes. Biotechnology 10, 121–135.

Kunamneni, A., Singh, S., (2006). Improved high thermal stability of pullulanase from a newly isolated thermophilic Bacillus sp. AN-7. Enzyme Microb. Technol. 39, 1399–1404. doi:10.1016/j.enzmictec.2006.03.023

Kuriki, T., Okada, S., Imanaka, T., (1988). New Type of Pullulanase from Bacillus stearothermophilus and Molecular Cloning and Expression of the Gene in Bacillus subtilis. J. Bacteriol. 170, 1554–1559.

Lambertz, C., Garvey, M., Klinger, J., Heesel, D., Klose, H., Fischer, R., Commandeur, U., (2014). Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol. Biofuels 7, 1–15. doi:10.1186/s13068-014-0135-5

Li, S., Xu, J., Bao, Y., Zheng, H., Song, H., (2015). Structure and sequence analysis-based engineering of pullulanase from Anoxybacillus sp. LM18-11 for improved thermostability. J. Biotechnol. 210, 8–14. doi:10.1016/j.jbiotec.2015.06.406

Li, X., Li, D., Park, K.-H., 2013. An extremely thermostable amylopullulanase from Staphylothermus marinus displays both pullulan- and cyclodextrin-degrading activities. Appl. Microbiol. Biotechnol. 97, 5359–5369. doi:10.1007/s00253-012-4397-1

Li, Y., Zhang, L., Ding, Z., Shi, G., 2013. Constitutive expression of a novel isoamylase from Bacillus lentus in Pichia pastoris for starch processing. Process Biochem. 48, 1303.

Lin, FP., Ho, YH., Lin, HY., Lin, HJ., (2012). Effect of C-terminal truncation on enzyme properties of recombinant amylopullulanase from Thermoanaerobacter pseudoethanolicus. Extremophiles 16, 395–403. doi:10.1007/s00792-012-0438-z

Lin, HY., Chuang, HH., Lin, FP., (2008). Biochemical characterization of engineered amylopullulanase from Thermoanaerobacter ethanolicus 39E-implicating the non-necessity of its 100 C-terminal amino acid residues. Extremophiles 12, 641–650. doi:10.1007/s00792-008-0168-4

Liu, G., Gu, Z., Hong, Y., Cheng, L., Li, C., (2017). Structure, functionality and applications of debranched starch: A review. Trends Food Sci. Technol. 63, 70–79. doi:10.1016/j.tifs.2017.03.004

Maalej, H., Hmidet, N., Ghorbel-Bellaaj, O., Nasri, M., (2013). Purification and biochemical characterization of a detergent stable alpha-amylase from Pseudomonas stutzeri AS22. Biotechnol. Bioprocess Eng. https://doi.org/10.1007/s12257-012-0862-z

Macauley-Patrick, S., Fazenda, M.L., McNeil, B., Harvey, L.M., (2005). Heterologous protein production using the Pichia pastoris expression system. Yeast 22, 249–270.

doi:10.1002/yea.1208

Market research report (2017). Biotechnology Market Analysis By Application (Health, Food & Agriculture, Natural Resources & Environment, Industrial Processing Bioinformatics), By Technology, And Segment Forecasts, 2018 - 2025. https://www.grandviewresearch.com/industry-analysis/biotechnology-market

Martin, C., Smith, A.M., (1995). Starch Biosynthesis. Plant Cell 7, 971–985. https://doi.org/10.1105/tpc.7.7.971

Matzke, J., Herrmann, A., Schneider, E., Bakker, EP., (2000). Gene cloning, nucleotide sequence and biochemical properties of a cytoplasmic cyclomaltodextrinase (neopullulanase) from Alicyclobacillus acidocaldarius, reclassification of a group of enzymes. FEMS Microbiol. Lett. 183, 55–61. https://doi.org/10.1111/j.1574-6968.2000.tb08933.x

McCleary, BV., Mangan, D., McKie, V., Cornaggia, C., Ivory, R., Rooney, E., (2014). Colourimetric and fluorometric substrates for measurement of pullulanase activity. Carbohydr. Res. 393, 60–69. doi:10.1016/j.carres.2014.04.014

Mercier, C., Frantz, B.M., Whelan, WJ., (1972). An improved purification of cell-bound pullulanase from Aerobacter aerogenes. Eur. J. Biochem. 26, 1–9. doi:10.1111/j.1432-1033.1972.tb01733.x

Miller, PS., and Blum, PH., (2010). Extremophile-inspired strategies for enzymatic biomass saccharification. Environ. Technol. 31, 1005–1015. https://doi.org/10.1080/09593330903536113

Morozkina, EV., Slutskaya, ES., Fedorova, TV, Tugay, TI., Golubeva, LI., Koroleva, OV, (2010). Extremophilic microorganisms: Biochemical adaptation and biotechnological application (review). Appl. Biochem. Microbiol. 46, 1–14. https://doi.org/10.1134/S0003683810010011

Nakano, A., Lee, C.Y., Yoshida, A., Matsumoto, T., Shiomi, N., Katoh, S., (2006). Effects of methanol feeding methods on chimeric α-amylase expression in continuous culture of Pichia pastoris. J. Biosci. Bioeng. 101, 227–231. doi:10.1263/jbb.101.227

Nelson, TE., and Larner, J., (1970). Studies on action of amylo-1,6-glucosidase. Biochim. Biophys. Acta - Enzymol. 198, 538–545.

Nguyen, HD., Nguyen, QA., Ferreira, RC., Ferreira, LCS., Tran, LT., Schumann, W., (2005). Construction of plasmid-based expression vectors for Bacillus subtilis exhibiting full structural stability. Plasmid 54, 241–248. doi:10.1016/j.plasmid.2005.05.001

Niehaus, F., Peters, A., Groudieva, T., Antranikian, G., (2000). Cloning, expression and biochemical characterisation of a unique thermostable pullulan-hydrolysing enzyme from the hyperthermophilic archaeon Thermococcus aggregans. FEMS Microbiol. Lett. 190, 223–229. doi:10.1016/S0378-1097(00)00339-6

Nielsen, J.E., Borchert, T. V, Vriend, G., (2001). The determinants of alpha-amylase pH-activity profiles. Protein Eng. 14, 505–512. https://doi.org/10.1093/protein/14.7.505

Nigam, P.S., (2013). Microbial enzymes with special characteristics for biotechnological applications. Biomolecules 3, 597–611.

Nisha, M., Satyanarayana, T., (2017). Characteristics and applications of recombinant

thermostable amylopullulanase of Geobacillus thermoleovorans secreted by Pichia pastoris. Appl. Microbiol. Biotechnol. 33, 2357–2369. doi:10.1007/s00253-016-8025-3

Nisha, M., Satyanarayana, T., (2013a). Characterization of recombinant amylopullulanase. Appl. Microbiol. Biotechnol. 97, 6279.

Nisha, M., Satyanarayana, T., (2013b). Characterization of recombinant amylopullulanase (gt-apu) and truncated amylopullulanase (gt-apuT) of the extreme thermophile Geobacillus thermoleovorans NP33 and their action in starch saccharification. Appl. Microbiol. Biotechnol. 97, 6279–6292. doi:10.1007/s00253-012-4538-6

Odibo, FJC., Obi, SKC., (1990). Optimum culture conditions for the production of the extracellular pullulanase of Thermoactinomyces thalpophilus. Biol. Wastes 32, 9–15. doi:10.1016/0269-7483(90)90068-4

Pantazaki, AA., Papaneophytou, CP., Lambropoulou, DA., (2011). Simultaneous polyhydroxyalkanoates and rhamnolipids production by Thermus thermophilus HB8. AMB Express 1, 1–13. https://doi.org/10.1186/2191-0855-1-17

Papaneophytou, CP., Pantazaki, AA., Kyriakidis, DA., (2009). An extracellular polyhydroxybutyrate depolymerase in Thermus thermophilus HB8. Appl. Microbiol. Biotechnol. 83, 659–668. https://doi.org/10.1007/s00253-008-1842-2

Pechan, T., Ma, PW., Luthe, DS., (2004). Heterologous expression of maize (Zea mays L) Mir1 cysteine proteinase in eukaryotic and prokaryotic expression systems. Prot Exp Pur 34, 134–141.

Persidis, A., (1998). Extremophiles. Nat. Biotechnol. 16, 593–594. https://doi.org/10.1038/nbt0698-593

Plant, AR., Morgan, HW., Daniel, RM., (1986). A highly stable pullulanase from Thermus aquaticus YT-1. Enzyme Microb. Technol. 8, 668–672. doi:10.1016/0141-0229(86)90063-3

Potvin, G., Ahmad, A., Zhang, Z., (2010). Bioprocess engineering aspects of heterologous protein production in Pichia pastoris: A review. Biochem. Eng. J. 64, 91. doi:10.1016/j.bej.2010.07.017

Ramesh, B., Reddy, PRM., Seenayya, G., Reddy, G., (2001). Effect of various flours on the production of thermostable β-amylase and pullulanase by Clostridium thermosulfurogenes SV2, Bioresource Technology. doi:10.1016/S0960-8524(00)00082-1

Behera, SS., Ray, RC., (2016). Solid state fermentation for production of microbial cellulases: Recent advances and improvement strategies. Int. J. Biol. Macromol. 86, 656–669.

Reddy, RM., Mrudula, S., Ramesh, B., Reddy, G., Seenayya, G., (2000). Production of thermostable pullulanase by Clostridium thermosulfurogenes SV2 in solid-state fermentation: optimization of enzyme leaching conditions using response surface methodology. Bioprocess Eng. 23, 0107–0112. doi:10.1007/s004499900129

Reddy, RM., Reddy, G., Seenayya, G., (1999a). Production of thermostable pullulanase by Clostridium thermosulfurogenes SV2 in solid-state fermentation: Optimization of nutrients levels using response surface methodology. Bioprocess Biosyst. Eng. 21, 497–503. doi:10.1007/PL00009088

Reddy, R.M., Reddy, P.G., Seenayya, G., (1999b). Enhanced production of thermostable β-amylase and pullulanase in the presence of surfactants by Clostridium thermosulfurogenes SV2. Process Biochem. 34, 87–92. doi:10.1016/S0032-9592(98)00073-9

Resina, D., Maurer, M., Cos, O., Arnau, C., Carnicer, M., Marx, H., Gasser, B., Valero, F., Mattanovich, D., Ferrer, P., (2009). Engineering of bottlenecks in Rhizopus oryzae lipase production in Pichia pastoris using the nitrogen source-regulated FLD1 promoter. N. Biotechnol. 25, 396–403. doi:10.1016/j.nbt.2009.01.008

Robyt, J.F., (2009). Starch: Chemistry and technology, Enzymes and their action on sarch, chapitre 7, in: BeMiller, J.N., Whistler, R.L. (Eds.), . London: Academic, p. 237.

Saha, BC., and Zeikus, JG., (1989). Novel highly thermostable pullulanase from thermophiles. Trends Biotechnol. 7, 234–239. doi:10.1016/0167-7799(89)90013-9

Satyanarayana, T., Littlechild, J., Kawarabayasi, Y., (2013). Thermophilic Microbes in Environmental and Industrial Biotechnology: Biotechnology of Thermophiles. Springer Netherlands, Dordrecht.

Schiraldi, C., De Rosa, M., (2002). The production of biocatalysts and biomolecules from extremophiles. Trends Biotechnol. https://doi.org/10.1016/S0167-7799(02)02073-5

Schiraldi, C., Marulli, F., Di Lernia, I., Martino, A., De Rosa, M., (1999). A microfiltration bioreactor to achieve high cell density in Sulfolobus solfataricus fermentation. Extremophiles 3, 199–204. doi:10.1007/s007920050117

Schülein, M., Højer-Pedersen, B., (1984). Characterization of new class of thermophilic pullulanases from Bacillus acidopullulyticus. Ann. N. Y. Acad. Sci. 434, 271–2714.

Shaw, J.-F., Lin, F.-P., Chen, S.-C., Chen, H.-C., (1995). Purification and properties of an extracellular α-amylase from Thermus sp. Bull. Acad. Sin 36, 195–200.

Song, W., Nie, Y., Mu, X.Q., Xu, Y., (2016). Enhancement of extracellular expression of Bacillus naganoensis pullulanase from recombinant Bacillus subtilis: Effects of promoter and host. Protein Expr. Purif. 124, 23–31. doi:10.1016/j.pep.2016.04.008

Su, Z., Lu, FP., Gao, Q., Liu, XG., Wang, BZ., Niu, T., (2010). Cloning and Expression of a Thermostable Pullulanase Gene from Thermotoga maritima MSB8 in Bacillus subtilis WB600, in: 2010 4th International Conference on Bioinformatics and Biomedical Engineering. IEEE, pp. 1–4. https://doi.org/10.1109/ICBBE.2010.5517795.

Street, T.O., Krukenberg, K.A., Rosgen, J., et al., (2010). Osmolyte-induced conformational changes in the Hsp90 molecular chaperone. Protein Sci.; 19(1): 57–65. doi: 10.1002/pro.282

Swamy, MV, Seenayya, G., (1996). Thermostable pullulanase and alpha-amylase activity from Clostridium thermosulfurogenes SV9 - Optmization of culture conditions for enzyme production. Process Biochem. 31(2), p. 157-162

Swarup, A., Lu, J., DeWoody, KC., Antoniewicz, MR., (2014). Metabolic network reconstruction, growth characterization and 13C-metabolic flux analysis of the extremophile Thermus thermophilus HB8. Metab. Eng. 24, 173. https://doi.org/10.1016/j.ymben.2014.05.013

Takami, H., Horikoshi, K., (2000). Analysis of the genome of an alkaliphilic Bacillus strain from an industrial point of view. Extremophiles 4, 99–108.

https://doi.org/10.1007/s007920050143

Takata, H., Kuriki, T., Okada, S., Takesada, Y., Iizuka, M., Minamiura, N., Imanaka, T., (1992). Action of neopullulanase. Neopullulanase catalyzes both hydrolysis and transglycosylation at alpha-(1-4)- and alpha-(1-6)-glucosidic linkages. J. Biol. Chem. 267, 18447.

Teague, W., Brumm, P., Allen, L., Brikun, I., (2001). Pulluanase expression constructs containing α-amylase promoter and leader sequences. Patent US6300115 B1.

Tomiyasu, K., Yato, K., Yasuda, M., Tonozuka, T., Ibuka, A., Sakai, H., (2001). Cloning and nucleotide sequence of the pullulanase gene of Thermus thermophilus HB8 and production of the enzyme in Escherichia coli . Biosci. Biotechnol. Biochem. 65, 2090–2094.

Turkenburg, JP., Brzozowski, AM., Svendsen, A., Borchert, T V, Davies, GJ., Wilson, KS., (2009). Structure of a pullulanase from Bacillus acidopullulyticus. Proteins 76, 516.

van den Burg, B., (2003). Extremophiles as a source for novel enzymes. Curr. Opin. Microbiol. 6, 213–218. https://doi.org/10.1016/S1369-5274(03)00060-2

van der Maarel, MJEC., van der Veen, B., Uitdehaag, JCM., Leemhuis, H., Dijkhuizen, L., (2002). Properties and applications of starch-converting enzymes of the alpha-amylase family. J. Biotechnol. 94, 137.

van Donkelaar, LHG., Mostert, J., Zisopoulos, F.K., Boom, R.M., van der Goot, AJ., (2016). The use of enzymes for beer brewing: Thermodynamic comparison on resource use. Energy 115, 519–527. doi:10.1016/j.energy.2016.09.011

Van Ee, J., Misset, O., Baas, E., (1997). Enzymes in detergency, Surfactant science series v 69. New York.

Varnavskaia OV, Illarionova NG, Selezneva AA, S.G., (1978). Effect of pH on the conformation properties and enzyme activity of alpha-amylase from Aspergillus terricola. Prikl Biokhim Mikrobiol. 14, 866–70.

Vázquez-Figueroa, E., Chaparro-Riggers, J., Bommarius, AS., (2007). Development of a thermostable glucose dehydrogenase by a structure-guided consensus concept. Chembiochem 8, 2295–2301.

Venturini, M., Morrione, A., Pisarra, P., Martegani, E., Vanoni, M., (1997). In Saccharomyces cerevisiae a short amino acid sequence facilitates excretion in the growth medium of periplasmic proteins. Mol. Microbiol. 23, 997–1007. doi:10.1046/j.1365-2958.1997.2841649.x

Vieille, C., Zeikus, GJ., (2001). Hyperthermophilic Enzymes: Sources, Uses, and Molecular Mechanisms for Thermostability. Microbiol. Mol. Biol. Rev. 65, 1–43. doi:10.1128/MMBR.65.1.1-43.2001

Wang, Y., Liu, Y., Wang, Z., Lu, F., (2014). Influence of promoter and signal peptide on the expression of pullulanase in Bacillus subtilis. Biotechnol. Lett. 36, 1783–1789. doi:10.1007/s10529-014-1538-x

Wu, H., Yu, X., Chen, L., Wu, G., (2014). Cloning, overexpression and characterization of a thermostable pullulanase from Thermus thermophilus HB27. Protein Expr. Purif. 95, 22–27. doi:10.1016/j.pep.2013.11.010

Xu, B., Yun-Juan, Y., Zun-Xi, H., (2006). cloning and over expression of gene encoding

pullulanase from Bacillus naganoensis in Pichia pastoris. J. Microbiol. Biotechnol. 16, 1185–1191.

Xu, J., Ren, F., Huang, C.H., Zheng, Y., Zhen, J., et al., (2013). Crystal structure of Pullulanase from *Anoxybacillus sp.* LM18-11. PDB. DOI: 10.2210/pdb3WDH/pdb

Xu, J., Ren, F., Huang, C., Zheng, Y., Zhen, J., Sun, H., Ko, T., He, M., Chen, C., Chan, H., Guo, R., Song, H., Ma, Y., (2014). Functional and structural studies of pullulanase from *Anoxybacillus sp.* LM18-11. Proteins Struct. Funct. Bioinforma. 82, 1685–1693. doi:10.1002/prot.24498

Yan, S., Wu, G., (2013). Secretory pathway of cellulase: a mini-review. Biotechnol Biofuels 6, 177.

Yang, M., (2013). Enhancement of heterologous expression of alkaline phytase in *Pichia pastoris*. ProQuest, UMI Dissertations Publishing.

Zhang, AL., Luo, JX., Zhang, TY., Pan, YW., Tan, YH., Fu, CY., Tu, F., (2009). Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. Mol. Biol. Rep. 36, 1611–1619. doi:10.1007/s11033-008-9359-4

Zhang, W., Inan, M., Meagher, MM., (2007). Rational design and optimization of fed-batch and continuous fermentations. Methods Mol. Biol. 389, 43–64. doi:10.1007/978-1-59745-456-8_4

Zou, C., Duan, X., Wu, J., (2014). Enhanced extracellular production of recombinant *Bacillus deramificans* pullulanase in *Escherichia coli* through induction mode optimization and a glycine feeding strategy. Bioresour. Technol. 172, 174–179.

Zouari Ayadi, D., Ben Ali, M., Jemli, S., Ben Mabrouk, S., Mezghani, M., Ben Messaoud, E., Bejar, S., (2008). Heterologous expression, secretion and characterization of the *Geobacillus thermoleovorans* US105 type I pullulanase. Appl. Microbiol. Biotechnol. 78, 473–481. doi:10.1007/s00253-007-1318-

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Thèse de doctorat
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